

UTILITY
PATENT APPLICATION

TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No. 8311

First Inventor or Application Identifier

Robert J. Isfort

Title

Methods For Identifying Compounds for Regulating
Muscle Mass or Function Using Vasoactive Intestinal
Peptide Receptors

Express Mail Label No.

EJ302199415US

APPLICATION ELEMENTS

See MPEP Chapter 600 concerning utility patent application contents.

ADDRESS TO:

Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 202311. ☒ * Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original, and a duplicate for fee processing)2. ☒ Specification Total Pages [54]
(preferred arrangement set forth below)

- Descriptive Title of the Invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R&D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3. ☒ Drawing(s) (35 USC 113) Total Sheets [10]

4. Oath or Declaration Total pages [2]

- a. ☒ Newly executed (original or copy) **Unsigned**
- b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 16 completed)

i. ☐ **DELETION OF INVENTORS**Signed statement attached deleting
inventor(s) named in the prior
application, see 37 CFR §§1.63(d)(2) and
1.33(b).*** NOTE FOR ITEMS 1 & 13: IN ORDER TO BE ENTITLED TO PAY
SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS
REQUIRED (37 C.F.R. §1.27), EXCEPT IF ONE FILED IN A PRIOR
APPLICATION IS RELIED UPON (37 C.F.R. §1.28).**5. ☐ Microfiche Computer Program (Appendix)6. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)

- a. ☒ Computer Readable copy
- b. ☒ Paper Copy (identical to computer copy)
- c. ☒ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

7. ☐ Assignment Papers (cover sheet & document(s))8. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)9. ☐ English Translation Document (if applicable)10. ☐ Information Disclosure ☐ Copies of IDS
Statement (IDS)/PTO-1449 Citations11. ☐ Preliminary Amendment12. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)13. ☐ *Small Entity ☐ Statement filed in prior application
Statement(s) Status still proper and desired14. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)15. ☐ Other:16. If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information below and in the preliminary amendment:☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No. 1

Prior application information: Examiner: _____ Group/Art Unit: _____

For **CONTINUATION** or **DIVISIONAL** only: The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

17. CORRESPONDENCE ADDRESS

☐ Customer Number or Bar Code Label(Insert Customer No. or Attach bar code label
here)or ☒ Correspondence address below

NAME

Cynthia M Bott

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8700 Mason-Montgomery Road

CITY

Mason

STATE

Ohio

ZIP CODE

45040

COUNTRY

USA

TELEPHONE

513-622-0087

FAX

513-622-3300

Name (Print/Type)

Cynthia M. Bott

Registration No. (Attorney/Agent)

46,568

Signature

Cynthia M Bott

Date

October 23, 2000

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

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**FEE TRANSMITTAL
for FY 2001**

Patent fees are subject to annual revision

Complete if Known

Application Number

Filing Date

October 23, 2000

First Named Inventor

Robert J. Isfort

Examiner Name

Group/Art Unit

TOTAL AMOUNT OF PAYMENT (\$ 1538.00)

Attorney Docket No..

8311

METHOD OF PAYMENT (check one)

- 1.
- ☒
- The Commissioner is hereby authorized to charge indicated fees and credit any over payments to:

Deposit Account Number **16-2480**Deposit Account Name **The Procter & Gamble Company**☒ Charge Any Additional Fee ☐ Applicant claims small entity status See 37 CFR §127
37 C.F.R. §§1.16 and 1.17

- 2.
- ☐
- Payment Enclosed:

☐ Check ☐ Credit Card ☐ Money Order ☐ Other**FEE CALCULATION****1. BASIC FILING FEE**

Large Entity Small Entity

Fee Code	Entity (\$)	Fee Code	Entity (\$)	Fee Description	Fee Paid
101	710	201	355	Utility filing fee	[710]
106	320	206	160	Design filing fee	
107	490	207	245	Plant filing fee	
108	710	208	355	Reissue filing fee	
114	150	214	75	Provisional filing fee	

SUBTOTAL (1) (\$)[710]**2. EXTRA CLAIM FEES**

Total Claims	Extra Claims	Fee From	Below	Fee Paid
Independent Claims	[26] - 20** = [6]	x	[18] =	[108]
Multiple Dependent	[12] - 3** = [9]	x	[80] =	[720]
			[0] =	[0]

** or number previously paid, if greater; For Reissues, see below

Large Fee Code	Entity (\$)	Small Fee Code	Entity (\$)	Fee Description
103	18	203	9	Claims in excess of 20
102	80	202	40	Independent claims in excess of 3
104	270	204	135	Multiple dependent claim, if not paid
109	80	209	40	**Reissue independent claims over original patent
110	18	210	9	**Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) (\$)[828]**FEE CALCULATION (continued)****3. ADDITIONAL FEES**

Large Fee Code	Entity (\$)	Small Fee Code	Entity (\$)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for ex parte reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner's action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner's action	
115	110	215	55	Extension for reply within 1 st month	
116	390	216	195	Extension for reply within 2 nd month	
117	890	217	445	Extension for reply within 3 rd month	
118	1,390	218	695	Extension for reply within 4 th month	
128	1,890	228	945	Extension for reply within 5 th month	
119	310	219	155	Notice of Appeal	
120	310	220	155	Filing a brief in support of an appeal	
121	270	221	135	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,240	241	620	Petition to revive - unintentional	
142	1,240	242	620	Utility issue fee (or reissue)	
143	440	243	220	Design issue fee	
144	600	244	300	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of IDS	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	710	246	355	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	710	249	355	For each additional invention to be examined (37 CFR § 1.129(b))	
179	710	279	355	Request for Continued Examination (RCE)	
169	710	249	355	Request for expedited examination of a design application	

Other fee (specify) _____

Other fee (specify) _____

* Reduced by Basic Filing Fee Paid **SUBTOTAL (3) (\$) [0]****SUBMITTED BY**

Name (Print/Type)

Cynthia M. Bott

Registration No.
(Attorney/Agent)

46,568

Complete (if applicable)

Telephone

(513) 622-0087

Signature

Cynthia M Bott

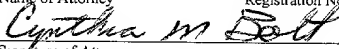
Date

October 23, 2000

WARNING: Information on this form may become public. Credit Card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, D.C. 20231.

"Express Mail" labeling number EJ302199415US
Date of Deposit October 23, 2000
I hereby certify that this paper or fee is being deposited with the
United States Postal "Express Mail Post Office to Addressee"
service under 37 CFR 1.10 on the date indicated above and is
addressed to Commissioner For Patents, Box CPA,
Washington, D.C. 20231

<u>Cynthia M. Bott</u>	<u>46,568</u>
Name of Attorney	Registration No.
	
Signature of Attorney	

P&G Case 8311

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of	:	
Robert J. Isfort and Russell J. Sheldon	:	Group Art Unit:
Serial No.:	:	Examiner:
Filed: October 23, 2000	:	

For: **METHODS FOR IDENTIFYING COMPOUNDS FOR
REGULATING MUSCLE MASS OR FUNCTION USING
VASOACTIVE INTESTINAL PEPTIDE RECEPTORS**

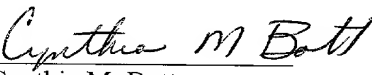
Statement under 37 CFR 1.82(f)

Box Sequence
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I hereby state that the information recorded in computer readable form is identical to the
written sequence.

Respectfully submitted,

By 
Cynthia M. Bott
Attorney for Applicants
Registration No. 46,568
(513) 622-0087

October 23, 2000
Cincinnati, Ohio
D:\cases\6138res.doc

SEQUENCE LISTING

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Sheldon, Russell

<120> Methods for Identifying Compounds for Regulating Muscle Mass or Function
Using Vasoactive Intestinal Peptide Receptors

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Gly	Val	Leu	Ala	Cys	Val	Leu	Gly	Pro	Val	Gly	Ser	Trp	Ala	Val	Gly		
		20						25					30				
Leu	Gln	Gln	Glu	Glu	Cys	Asp	Tyr	Leu	Gln	Met	Ile	Lys	Val	Gln	His		
		35					40					45					
Lys	Gln	Cys	Leu	Glu	Glu	Ala	Gln	Leu	Glu	Asn	Glu	Thr	Ser	Gly	Cys		
		50				55					60						
Ser	Lys	Met	Trp	Asp	Asn	Leu	Thr	Cys	Trp	Pro	Ala	Thr	Pro	Arg	Gly		
65					70					75					80		
Gln	Val	Val	Val	Leu	Ala	Cys	Pro	Leu	Ile	Phe	Lys	Leu	Phe	Ser	Pro		
				85					90					95			
Thr	Gln	Gly	Leu	Asn	Val	Ser	Arg	Asn	Cys	Thr	Asp	Glu	Gly	Trp	Thr		
			100					105					110				
Pro	Leu	Glu	Pro	Gly	Pro	Tyr	Pro	Ile	Ala	Cys	Gly	Met	Asp	Asp	Lys		
		115					120					125					
Ala	Ser	Gly	Leu	Asp	Glu	Gln	Gln	Thr	Val	Phe	Tyr	Asn	Ser	Val	Lys		
		130				135						140					
Thr	Gly	Tyr	Thr	Ile	Gly	Tyr	Ser	Leu	Ser	Leu	Ala	Ala	Leu	Leu	Val		
145					150					155					160		
Ala	Thr	Ala	Ile	Leu	Ser	Leu	Phe	Arg	Lys	Leu	His	Cys	Thr	Arg	Asn		
				165					170					175			
Tyr	Ile	His	Met	His	Leu	Phe	Ile	Ser	Phe	Ile	Leu	Arg	Ala	Thr	Ala		
			180					185					190				
Val	Phe	Ile	Lys	Asp	Leu	Ala	Leu	Phe	Asp	Ser	Glu	Glu	Ser	Asp	His		
		195					200					205					
Cys	Ser	Lys	Gly	Ser	Val	Gly	Cys	Lys	Ala	Ala	Val	Val	Leu	Phe	Gln		
		210				215					220						
Tyr	Cys	Val	Met	Ala	Asn	Phe	Phe	Trp	Leu	Leu	Val	Glu	Gly	Leu	Tyr		
225					230					235					240		
Leu	His	Thr	Leu	Leu	Ala	Val	Ser	Phe	Phe	Ser	Glu	Arg	Lys	Tyr	Phe		
				245					250					255			
Trp	Gly	Tyr	Ile	Phe	Val	Gly	Trp	Gly	Val	Pro	Ser	Thr	Phe	Ile	Met		
			260					265					270				
Val	Trp	Thr	Val	Val	Arg	Ile	His	Phe	Glu	Asp	Tyr	Gly	Cys	Trp	Asp		
			275				280					285					
Thr	Ile	His	Ser	Ser	Leu	Trp	Trp	Ile	Ile	Lys	Ala	Pro	Ile	Leu	Ala		
					295						300						
Ser	Ile	Leu	Val	Asn	Phe	Ile	Leu	Phe	Ile	Arg	Ile	Ile	Gly	Ile	Leu		
305					310					315					320		

Val Gln Lys Leu Arg Pro Pro Asp Val Gly Lys Ser Asp Asn Ser Pro
325 330 335

Tyr Ser Arg Leu Ala Lys Ser Thr Leu Leu Leu Ile Pro Leu Phe Gly
340 345 350

Val His Tyr Ile Met Phe Ala Phe Phe Pro Asp Asn Phe Lys Ala Glu
355 360 365

Val Lys Met Val Phe Glu Leu Ile Val Gly Ser Phe Gln Gly Cys Val
370 375 380

Val Ala Ile Leu Tyr Cys Phe Leu Asn Gly Glu Val Gln Ala Glu Leu
385 390 395 400

Arg Arg Lys Trp Arg Arg Trp His Gln Gln Gly Val Leu Gly Trp Asp
405 410 415

Ser Lys Tyr Gln His Pro Ser Gly Gly Ser Asn Gly Asp Thr Cys Ser
420 425 430

Thr Gln Val Ser Met Leu Thr Arg Val Ser Pro Ser Ala Arg Arg Ser
435 440 445

Ser Ser Phe Gln Ala Glu Val Ser Leu Val
450 455

<210> 6

<211> 444

<212> PRT

<213> rana ridibunda;

<400> 6

Met Glu Phe Leu Pro Leu Leu Leu Cys Leu Thr Gly Leu Phe Ser Pro
1 5 10 15

Ile Leu Cys Val Pro Glu Glu Cys Ser Ile Met Tyr Gln Ile Glu Leu
20 25 30

Lys His Glu Glu Cys Val Asn His Glu Asp Tyr Phe Asn Asp Thr Ala
35 40 45

Val Cys Lys Arg Thr Trp Asp Asn Ile Thr Cys Trp Pro Ser Ala Ser
50 55 60

Ile Gly Glu Val Val Val Leu Gln Cys Pro Gly Tyr Phe Ser Met Phe
65 70 75 80

Thr Thr Gly Thr Val Asn Gly Asn Val Ser Lys Asn Cys Thr Ser Glu
85 90 95

Gly Trp Ser Glu Met Tyr Pro Ala Thr Tyr Ala Ala Ala Cys Gly Phe
100 105 110

Ser Thr Asn Asp Thr Pro Thr Glu Gln Gln Thr Val Phe Phe Gly Ala

115					120					125					
Ile	Lys	Thr	Gly	Tyr	Thr	Ile	Gly	His	Ser	Leu	Ser	Leu	Ile	Ser	Leu
	130						135					140			
Thr	Ala	Ala	Met	Ile	Ile	Leu	Cys	Ile	Phe	Arg	Lys	Leu	His	Cys	Thr
	145						150					155			160
Arg	Asn	Tyr	Ile	His	Met	His	Leu	Phe	Met	Ser	Phe	Ile	Met	Arg	Ala
					165					170					175
Ile	Ala	Val	Phe	Ile	Lys	Asp	Ile	Val	Leu	Phe	Glu	Ser	Gly	Glu	Ser
					180					185					190
Asp	His	Cys	His	Val	Gly	Ser	Val	Gly	Cys	Lys	Ala	Ala	Met	Val	Phe
		195								200					205
Phe	Gln	Tyr	Cys	Ile	Met	Ala	Asn	Phe	Phe	Trp	Leu	Leu	Val	Glu	Gly
		210								220					
Leu	Tyr	Leu	His	Asn	Leu	Leu	Val	Ile	Ser	Phe	Phe	Ser	Glu	Lys	Lys
		225								235					240
Tyr	Phe	Trp	Trp	Tyr	Ile	Leu	Ile	Gly	Trp	Gly	Ala	Pro	Ser	Val	Phe
					245					250					255
Ile	Thr	Ala	Trp	Ser	Leu	Ala	Arg	Val	Tyr	Phe	Glu	Asp	Thr	Gly	Cys
					260					265					270
Trp	Asp	Thr	Ile	Glu	Ser	His	Leu	Trp	Trp	Ile	Ile	Lys	Thr	Pro	Ile
					275					280					285
Leu	Val	Ser	Ile	Leu	Val	Asn	Phe	Ile	Leu	Phe	Ile	Cys	Ile	Ile	Arg
					290					300					
Ile	Leu	Val	Gln	Lys	Leu	His	Ser	Pro	Asp	Val	Gly	Arg	Asn	Glu	Asn
					305					315					320
Ser	Gln	Tyr	Thr	Arg	Leu	Ala	Lys	Ser	Thr	Leu	Leu	Leu	Ile	Pro	Leu
					325					330					335
Phe	Gly	Val	His	Tyr	Ile	Met	Phe	Ala	Phe	Phe	Pro	Asp	Asn	Phe	Lys
					340					345					350
Val	Glu	Val	Lys	Leu	Val	Phe	Glu	Leu	Ile	Leu	Gly	Ser	Phe	Gln	Gly
					355					360					365
Phe	Val	Val	Ala	Val	Leu	Tyr	Cys	Phe	Leu	Asn	Gly	Glu	Val	Gln	Ala
					370					375					380
Glu	Leu	Lys	Arg	Lys	Trp	Arg	Arg	Trp	Asn	Leu	Glu	Arg	Phe	Met	Gly
					385					390					400
Lys	Asp	Met	Lys	Tyr	His	His	Pro	Ser	Leu	Gly	Ser	Asn	Gly	Thr	Asn
					405					410					415
Phe	Ser	Thr	Gln	Ile	Ser	Met	Leu	Thr	Lys	Cys	Ser	Pro	Lys	Thr	Arg

420 425 430
 Arg Cys Ser Ser Phe Gln Ala Glu Phe Ser Leu Val
 435 440

 <210> 7
 <211> 458
 <212> PRT
 <213> porcine;

 <400> 7

 Met Arg Pro Leu Ser Pro Pro Pro Ala Gly Trp Phe Cys Val Leu Ala
 1 5 10 15

 Gly Val Leu Ala Cys Val Leu Gly Pro Val Gly Ser Trp Ala Val Gly
 20 25 30

 Leu Gln Gln Glu Glu Cys Asp Tyr Leu Gln Met Ile Lys Val Gln His
 35 40 45

 Lys Gln Cys Leu Glu Glu Ala Gln Leu Glu Asn Glu Thr Ser Gly Cys
 50 55 60

 Ser Lys Met Trp Asp Asn Leu Thr Cys Trp Pro Ala Thr Pro Arg Gly
 65 70 75 80

 Gln Val Val Val Leu Ala Cys Pro Leu Ile Phe Lys Leu Phe Ser Pro
 85 90 95

 Thr Gln Gly Leu Asn Val Ser Arg Asn Cys Thr Asp Glu Gly Trp Thr
 100 105 110

 Pro Leu Glu Pro Gly Pro Tyr Pro Ile Ala Cys Gly Met Asp Asp Lys
 115 120 125

 Ala Ser Gly Leu Asp Glu Gln Gln Thr Val Phe Tyr Asn Ser Val Lys
 130 135 140

 Thr Gly Tyr Thr Ile Gly Tyr Ser Leu Ser Leu Ala Ala Leu Leu Val
 145 150 155 160

 Ala Thr Ala Ile Leu Ser Leu Phe Arg Lys Leu His Cys Thr Arg Asn
 165 170 175

 Tyr Ile His Met His Leu Phe Ile Ser Phe Ile Leu Arg Ala Thr Ala
 180 185 190

 Val Phe Ile Lys Asp Leu Ala Leu Phe Asp Ser Glu Glu Ser Asp His
 195 200 205

 Cys Ser Lys Gly Ser Val Gly Cys Lys Ala Ala Val Val Leu Phe Gln
 210 215 220

 Tyr Cys Val Met Ala Asn Phe Phe Trp Leu Leu Val Glu Gly Leu Tyr
 225 230 235 240

Leu His Thr Leu Leu Ala Val Ser Phe Phe Ser Glu Arg Lys Tyr Phe
 245 250 255
 Trp Gly Tyr Ile Phe Val Gly Trp Gly Val Pro Ser Thr Phe Ile Met
 260 265 270
 Val Trp Thr Val Val Arg Ile His Phe Glu Asp Tyr Gly Cys Trp Asp
 275 280 285
 Thr Ile His Ser Ser Leu Trp Trp Ile Ile Lys Ala Pro Ile Leu Ala
 290 295 300
 Ser Ile Leu Val Asn Phe Ile Leu Phe Ile Arg Ile Ile Gly Ile Leu
 305 310 315 320
 Val Gln Lys Leu Arg Pro Pro Asp Val Gly Lys Ser Asp Asn Ser Pro
 325 330 335
 Tyr Ser Arg Leu Ala Lys Ser Thr Leu Leu Leu Ile Pro Leu Phe Gly
 340 345 350
 Val His Tyr Ile Met Phe Ala Phe Phe Pro Asp Asn Phe Lys Ala Glu
 355 360 365
 Val Lys Met Val Phe Glu Leu Ile Val Gly Ser Phe Gln Gly Cys Val
 370 375 380
 Val Ala Ile Leu Tyr Cys Phe Leu Asn Gly Glu Val Gln Ala Glu Leu
 385 390 395 400
 Arg Arg Lys Trp Arg Arg Trp His Gln Gln Gly Val Leu Gly Trp Asp
 405 410 415
 Ser Lys Tyr Gln His Pro Ser Gly Gly Ser Asn Gly Asp Thr Cys Ser
 420 425 430
 Thr Gln Val Ser Met Leu Thr Arg Val Ser Pro Ser Ala Arg Arg Ser
 435 440 445
 Ser Ser Phe Gln Ala Glu Val Ser Leu Val
 450 455

<210> 8
 <211> 459
 <212> PRT
 <213> rattus sp;

<400> 8

Met Arg Pro Pro Ser Pro Pro His Val Arg Trp Leu Cys Val Leu Ala
 1 5 10 15
 Gly Ala Leu Ala Cys Ala Leu Arg Pro Ala Gly Ser Gln Ala Ala Ser
 20 25 30
 Pro Gln His Glu Cys Glu Tyr Leu Gln Leu Ile Glu Ile Gln Arg Gln
 35 40 45

Gln	Cys	Leu	Glu	Glu	Ala	Gln	Leu	Glu	Asn	Glu	Thr	Thr	Gly	Cys	Ser	50	55	60
Lys	Met	Trp	Asp	Asn	Leu	Thr	Cys	Trp	Pro	Thr	Thr	Pro	Arg	Gly	Gln	65	70	75
Ala	Val	Val	Leu	Asp	Cys	Pro	Leu	Ile	Phe	Gln	Leu	Phe	Ala	Pro	Ile	85	90	95
His	Gly	Tyr	Asn	Ile	Ser	Arg	Ser	Cys	Thr	Glu	Glu	Gly	Trp	Ser	Gln	100	105	110
Leu	Glu	Pro	Gly	Pro	Tyr	His	Ile	Ala	Cys	Gly	Leu	Asn	Asp	Arg	Ala	115	120	125
Ser	Ser	Leu	Asp	Glu	Gln	Gln	Gln	Thr	Lys	Phe	Tyr	Asn	Thr	Val	Lys	130	135	140
Thr	Gly	Tyr	Thr	Ile	Gly	Tyr	Ser	Leu	Ser	Leu	Ala	Ser	Leu	Leu	Val	145	150	155
Ala	Met	Ala	Ile	Leu	Ser	Leu	Phe	Arg	Lys	Leu	His	Cys	Thr	Arg	Asn	165	170	175
Tyr	Ile	His	Met	His	Leu	Phe	Met	Ser	Phe	Ile	Leu	Arg	Ala	Thr	Ala	180	185	190
Val	Phe	Ile	Lys	Asp	Met	Ala	Leu	Phe	Asn	Ser	Gly	Glu	Ile	Asp	His	195	200	205
Cys	Ser	Glu	Ala	Ser	Val	Gly	Cys	Lys	Ala	Ala	Val	Val	Phe	Phe	Gln	210	215	220
Tyr	Cys	Val	Met	Ala	Asn	Phe	Phe	Trp	Leu	Leu	Val	Glu	Gly	Leu	Tyr	225	230	235
Leu	Tyr	Thr	Leu	Leu	Ala	Val	Ser	Phe	Phe	Ser	Glu	Arg	Lys	Tyr	Phe	245	250	255
Trp	Gly	Tyr	Ile	Leu	Ile	Gly	Trp	Gly	Val	Pro	Ser	Val	Phe	Ile	Thr	260	265	270
Ile	Trp	Thr	Val	Val	Arg	Ile	Tyr	Phe	Glu	Asp	Phe	Gly	Cys	Trp	Asp	275	280	285
Thr	Ile	Ile	Asn	Ser	Ser	Leu	Trp	Trp	Ile	Ile	Lys	Ala	Pro	Ile	Leu	290	295	300
Leu	Ser	Ile	Leu	Val	Asn	Phe	Val	Leu	Phe	Ile	Cys	Ile	Ile	Arg	Ile	305	310	315
Leu	Val	Gln	Lys	Leu	Arg	Pro	Pro	Asp	Ile	Gly	Lys	Asn	Asp	Ser	Ser	325	330	335
Pro	Tyr	Ser	Arg	Leu	Ala	Lys	Ser	Thr	Leu	Leu	Leu	Ile	Pro	Leu	Phe	340	345	350

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Gly Ile His Tyr Val Met Phe Ala Phe Phe Pro Asp Asn Phe Lys Ala
355 360 365

Gln Val Lys Met Val Phe Glu Leu Val Val Gly Ser Phe Gln Gly Phe
370 375 380

Val Val Ala Ile Leu Tyr Cys Phe Leu Asn Gly Glu Val Gln Ala Glu
385 390 395 400

Leu Arg Arg Lys Trp Arg Arg Trp His Leu Gln Gly Val Leu Gly Trp
405 410 415

Ser Ser Lys Ser Gln His Pro Trp Gly Gly Ser Asn Gly Ala Thr Cys
420 425 430

Ser Thr Gln Val Ser Met Leu Thr Arg Val Ser Pro Ser Ala Arg Arg
435 440 445

Ser Ser Ser Phe Gln Ala Glu Val Ser Leu Val
450 455

<210> 9

<211> 447

<212> PRT

<213> Carassius auratus;

<400> 9

Met Cys Asp Val Val Asn Glu Ile Glu Leu Ala Arg Ala Arg Cys Glu
1 5 10 15

Asn Lys Thr Ala Gly Asn Val Thr Ser Gly Cys Lys Gly Met Trp Asp
20 25 30

Ile Ile Ala Cys Trp Pro Ser Ala Lys Val Gly Glu His Val Val Ile
35 40 45

Pro Cys Pro Asn Tyr Phe Arg His Phe Ser Asp His His Glu Gly Asn
50 55 60

Leu Ser Lys Thr Cys Thr Ala Asp Gly Trp Thr Glu Met Asp Pro Met
65 70 75 80

Glu Ile Ala Val Tyr Cys Gly Tyr Asn Leu Asn Gly Thr Val Asp Asp
85 90 95

Asp Ser Phe Phe Arg Ser Val Lys Ile Gly Tyr Thr Ile Gly His Ser
100 105 110

Val Ser Leu Ile Ser Leu Thr Thr Ala Ile Val Ile Leu Cys Met Ser
115 120 125

Arg Lys Leu His Cys Thr Arg Asn Tyr Ile His Met His Leu Phe Val
130 135 140

Ser Phe Ile Leu Lys Ala Ile Ala Val Phe Val Lys Asp Ala Val Leu

145		150		155		160
Tyr Asp Val Ile Gln Glu Ser Asp Asn Cys Ser Thr Ala Ser Val Gly						
	165			170		175
Cys Lys Ala Val Ile Val Phe Phe Gln Tyr Cys Ile Met Ala Ser Phe						
	180			185		190
Phe Trp Leu Leu Val Glu Gly Leu Tyr Leu His Ala Leu Leu Ala Val						
	195			200		205
Ser Phe Phe Ser Glu Arg Lys Tyr Phe Trp Trp Tyr Ile Leu Ile Gly						
	210			215		220
Trp Gly Gly Pro Thr Ile Phe Ile Met Ala Trp Ser Phe Ala Lys Ala						
	225			230		235
Tyr Phe Asn Asp Val Gly Cys Trp Asp Ile Ile Glu Asn Ser Asp Leu						
	245			250		255
Phe Trp Trp Ile Ile Lys Thr Pro Ile Leu Ala Ser Ile Leu Met Asn						
	260			265		270
Phe Ile Leu Phe Ile Cys Ile Ile Arg Ile Leu Arg Gln Lys Ile Asn						
	275			280		285
Cys Pro Asp Ile Gly Arg Asn Glu Ser Asn Gln Tyr Ser Arg Leu Ala						
	290			295		300
Lys Ser Thr Leu Leu Leu Ile Pro Leu Phe Gly Ile Asn Phe Ile Ile						
	305			310		315
Phe Ala Phe Ile Pro Glu Asn Ile Lys Thr Glu Leu Arg Leu Val Phe						
	325			330		335
Asp Leu Ile Leu Gly Ser Phe Gln Gly Phe Val Val Ala Val Leu Tyr						
	340			345		350
Cys Phe Leu Asn Gly Glu Val Gln Ala Glu Ile Lys Arg Lys Trp Arg						
	355			360		365
Arg Trp His Leu Glu Arg Phe Leu Gly Pro Asp Thr Lys Tyr Gln His						
	370			375		380
Pro Ser Met Gly Ser Asn Gly Asn Asn Phe Ser Thr Gln Ile Ser Met						
	385			390		395
Leu Thr Arg Cys Ser Pro Lys Thr Arg Arg Ala Ser Thr Cys Gln Asp						
	405			410		415
Glu Thr Ser Ile Thr Val Leu Gly Ser Thr Thr Met Gly Tyr Gly His						
	420			425		430
Gln Asn Glu Thr Val Lys Gly His Glu Asp Val Arg Glu Val Ser						
	435			440		445

<210> 10

<211> 438
<212> PRT
<213> homo sapiens;

<400> 10

Met	Arg	Thr	Leu	Leu	Pro	Pro	Ala	Leu	Leu	Thr	Cys	Trp	Leu	Leu	Ala	
1			5					10					15			
Pro	Val	Asn	Ser	Ile	His	Pro	Glu	Cys	Arg	Phe	His	Leu	Glu	Ile	Gln	
		20					25					30				
Glu	Glu	Glu	Thr	Lys	Cys	Ala	Glu	Leu	Leu	Arg	Ser	Gln	Thr	Glu	Lys	
		35					40					45				
His	Lys	Ala	Cys	Ser	Gly	Val	Trp	Asp	Asn	Ile	Thr	Cys	Trp	Arg	Pro	
	50					55					60					
Ala	Asn	Val	Gly	Glu	Thr	Val	Thr	Val	Pro	Cys	Pro	Lys	Val	Phe	Ser	
65					70					75					80	
Asn	Phe	Tyr	Ser	Lys	Ala	Gly	Asn	Ile	Ser	Lys	Asn	Cys	Thr	Ser	Asp	
			85					90						95		
Gly	Trp	Ser	Glu	Thr	Phe	Pro	Asp	Phe	Val	Asp	Ala	Cys	Gly	Tyr	Ser	
		100						105					110			
Asp	Pro	Glu	Asp	Glu	Ser	Lys	Ile	Thr	Phe	Tyr	Ile	Leu	Val	Lys	Ala	
		115					120					125				
Ile	Tyr	Thr	Leu	Gly	Tyr	Ser	Val	Ser	Leu	Met	Ser	Leu	Ala	Thr	Gly	
	130					135					140					
Ser	Ile	Ile	Leu	Cys	Leu	Phe	Arg	Lys	Leu	His	Cys	Thr	Arg	Asn	Tyr	
145					150					155					160	
Ile	His	Leu	Asn	Leu	Phe	Leu	Ser	Phe	Ile	Leu	Arg	Ala	Ile	Ser	Val	
			165						170					175		
Leu	Val	Lys	Asp	Asp	Val	Leu	Tyr	Ser	Ser	Ser	Gly	Thr	Leu	His	Cys	
		180						185					190			
Pro	Asp	Gln	Pro	Ser	Ser	Trp	Val	Gly	Cys	Lys	Leu	Ser	Leu	Val	Phe	
		195					200					205				
Leu	Gln	Tyr	Cys	Ile	Met	Ala	Asn	Phe	Phe	Trp	Leu	Leu	Val	Glu	Gly	
	210					215					220					
Leu	Tyr	Leu	His	Thr	Leu	Leu	Val	Ala	Met	Leu	Pro	Pro	Arg	Arg	Cys	
225					230					235					240	
Phe	Leu	Ala	Tyr	Leu	Leu	Ile	Gly	Trp	Gly	Leu	Pro	Thr	Val	Cys	Ile	
			245						250					255		
Gly	Ala	Trp	Thr	Ala	Ala	Arg	Leu	Tyr	Leu	Glu	Asp	Thr	Gly	Cys	Trp	
			260					265					270			

Asp Thr Asn Asp His Ser Val Pro Trp Trp Val Ile Arg Ile Pro Ile
275 280 285

Leu Ile Ser Ile Ile Val Asn Phe Val Leu Phe Ile Ser Ile Ile Arg
290 295 300

Ile Leu Leu Gln Lys Leu Thr Ser Pro Asp Val Gly Gly Asn Asp Gln
305 310 315 320

Ser Gln Tyr Lys Arg Leu Ala Lys Ser Thr Leu Leu Leu Ile Pro Leu
325 330 335

Phe Gly Val His Tyr Met Val Phe Ala Val Phe Pro Ile Ser Ile Ser
340 345 350

Ser Lys Tyr Gln Ile Leu Phe Glu Leu Cys Leu Gly Ser Phe Gln Gly
355 360 365

Leu Val Val Ala Val Leu Tyr Cys Phe Leu Asn Ser Glu Val Gln Cys
370 375 380

Glu Leu Lys Arg Lys Trp Arg Ser Arg Cys Pro Thr Pro Ser Ala Ser
385 390 395 400

Arg Asp Tyr Arg Val Cys Gly Ser Ser Phe Ser Arg Asn Gly Ser Glu
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Glu Glu Glu Thr Lys Cys Thr Glu Leu Leu Arg Ser Gln Thr Glu Lys
35 40 45

His Lys Ala Cys Ser Gly Val Trp Asp Asn Ile Thr Cys Trp Arg Pro
50 55 60

Ala Asn Val Gly Glu Thr Val Thr Val Pro Cys Pro Lys Val Phe Ser
65 70 75 80

Asn Phe Tyr Ser Lys Ala Gly Asn Ile Ser Lys Asn Cys Thr Ser Asp
85 90 95

Gly	Trp	Ser	Glu	Thr	Phe	Pro	Asp	Phe	Val	Asp	Ala	Cys	Gly	Tyr	Ser	100	105	110
Asp	Pro	Glu	Asp	Glu	Ser	Lys	Ile	Thr	Phe	Tyr	Ile	Leu	Val	Lys	Ala	115	120	125
Ile	Tyr	Thr	Leu	Gly	Tyr	Ser	Val	Ser	Leu	Met	Ser	Leu	Ala	Thr	Gly	130	135	140
Ser	Ile	Ile	Leu	Cys	Leu	Phe	Arg	Lys	Leu	His	Cys	Thr	Arg	Asn	Tyr	145	150	155
Ile	His	Leu	Asn	Leu	Phe	Leu	Ser	Phe	Ile	Leu	Arg	Ala	Ile	Ser	Val	165	170	175
Leu	Val	Lys	Asp	Asp	Val	Leu	Tyr	Ser	Ser	Ser	Gly	Thr	Leu	His	Cys	180	185	190
Pro	Asp	Gln	Pro	Ser	Ser	Trp	Val	Gly	Cys	Lys	Leu	Ser	Leu	Val	Phe	195	200	205
Leu	Gln	Tyr	Cys	Ile	Met	Ala	Asn	Phe	Phe	Trp	Leu	Leu	Val	Glu	Gly	210	215	220
Leu	Tyr	Leu	His	Thr	Leu	Leu	Val	Ala	Met	Leu	Pro	Pro	Arg	Arg	Cys	225	230	235
Phe	Leu	Ala	Tyr	Leu	Leu	Ile	Gly	Trp	Gly	Leu	Pro	Thr	Val	Cys	Ile	245	250	255
Gly	Ala	Trp	Thr	Ala	Ala	Arg	Leu	Tyr	Leu	Glu	Asp	Thr	Gly	Cys	Trp	260	265	270
Asp	Thr	Asn	Asp	His	Ser	Val	Pro	Trp	Trp	Val	Ile	Arg	Ile	Pro	Ile	275	280	285
Leu	Ile	Ser	Ile	Ile	Val	Asn	Phe	Val	Leu	Phe	Ile	Ser	Ile	Ile	Arg	290	295	300
Ile	Leu	Leu	Gln	Lys	Leu	Thr	Ser	Pro	Asp	Val	Gly	Gly	Asn	Asp	Gln	305	310	315
Ser	Gln	Tyr	Lys	Arg	Leu	Ala	Lys	Ser	Thr	Leu	Leu	Leu	Ile	Pro	Leu	325	330	335
Phe	Gly	Val	His	Tyr	Met	Val	Phe	Ala	Val	Phe	Pro	Ile	Ser	Ile	Ser	340	345	350
Ser	Lys	Tyr	Gln	Ile	Leu	Phe	Glu	Leu	Cys	Leu	Gly	Ser	Phe	Gln	Gly	355	360	365
Leu	Val	Val	Ala	Val	Leu	Tyr	Cys	Phe	Leu	Asn	Ser	Glu	Val	Gln	Cys	370	375	380
Glu	Leu	Lys	Arg	Lys	Trp	Arg	Ser	Arg	Cys	Pro	Thr	Pro	Ser	Ala	Ser	385	390	395
																		400

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35 40 45

His Lys Ala Cys Ser Gly Val Trp Asp Asn Ile Thr Cys Trp Arg Pro
50 55 60

Ala Asn Val Gly Glu Thr Val Thr Val Pro Cys Pro Lys Val Phe Ser
65 70 75 80

Asn Phe Tyr Ser Lys Ala Gly Asn Ile Ser Lys Asn Cys Thr Ser Asp
85 90 95

Gly Trp Ser Glu Thr Phe Pro Asp Phe Val Asp Ala Cys Gly Tyr Ser
100 105 110

Asp Pro Glu Asp Glu Ser Lys Ile Thr Phe Tyr Ile Leu Val Lys Ala
115 120 125

Ile Tyr Thr Leu Gly Tyr Ser Val Ser Leu Met Ser Leu Ala Thr Gly
130 135 140

Ser Ile Ile Leu Cys Leu Phe Arg Lys Leu His Cys Thr Arg Asn Tyr
145 150 155 160

Ile His Leu Asn Leu Phe Leu Ser Phe Ile Leu Arg Ala Ile Ser Val
165 170 175

Leu Val Lys Asp Asp Val Leu Tyr Ser Ser Ser Gly Thr Leu His Cys
180 185 190

Pro Asp Gln Pro Ser Ser Trp Val Gly Cys Lys Leu Ser Leu Val Phe
195 200 205

Leu Gln Tyr Cys Ile Met Ala Asn Phe Phe Trp Leu Leu Val Glu Gly

210	215	220
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Phe Leu Ala Tyr Leu Leu Ile Gly Trp Gly Leu Pro Thr Val Cys Ile 245 250 255		
Gly Ala Trp Thr Ala Ala Arg Leu Tyr Leu Glu Asp Thr Gly Cys Trp 260 265 270		
Asp Thr Asn Asp His Ser Val Pro Trp Trp Val Ile Arg Ile Pro Ile 275 280 285		
Leu Ile Ser Ile Ile Val Asn Phe Val Leu Phe Ile Ser Ile Ile Arg 290 295 300		
Ile Leu Leu Gln Lys Leu Thr Ser Pro Asp Val Gly Gly Asn Asp Gln 305 310 315 320		
Ser Gln Tyr Lys Arg Leu Ala Lys Ser Thr Leu Leu Leu Ile Pro Leu 325 330 335		
Phe Gly Val His Tyr Met Val Phe Ala Val Phe Pro Ile Ser Ile Ser 340 345 350		
Ser Lys Tyr Gln Ile Leu Phe Glu Leu Cys Leu Gly Ser Phe Gln Gly 355 360 365		
Leu Val Val Ala Val Leu Tyr Cys Phe Leu Asn Ser Glu Val Gln Cys 370 375 380		
Glu Leu Lys Arg Lys Trp Arg Ser Arg Cys Pro Thr Pro Ser Ala Ser 385 390 395 400		
Arg Asp Tyr Arg Val Cys Gly Ser Ser Phe Ser His Asn Gly Ser Glu 405 410 415		
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Arg	Ala	Cys	Ser	Gly	Val	Trp	Asp	Asn	Ile	Thr	Cys	Trp	Arg	Pro	Ala		
	50					55					60						
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Phe	Tyr	Ser	Arg	Pro	Gly	Asn	Ile	Ser	Lys	Asn	Cys	Thr	Ser	Asp	Gly		
			85					90						95			
Trp	Ser	Glu	Thr	Phe	Pro	Asp	Phe	Ile	Asp	Ala	Cys	Gly	Tyr	Asn	Asp		
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Pro	Glu	Asp	Glu	Ser	Lys	Ile	Ser	Phe	Tyr	Ile	Leu	Val	Lys	Ala	Ile		
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		130				135						140					
Ile	Ile	Ile	Cys	Leu	Phe	Arg	Lys	Leu	His	Cys	Thr	Arg	Asn	Tyr	Ile		
145					150				155						160		
His	Leu	Asn	Leu	Phe	Leu	Ser	Phe	Met	Leu	Arg	Ala	Ile	Ser	Val	Leu		
			165					170						175			
Val	Lys	Asp	Ser	Val	Leu	Tyr	Ser	Ser	Ser	Gly	Leu	Leu	Arg	Cys	His		
			180					185					190				
Asp	Gln	Pro	Ala	Ser	Trp	Val	Gly	Cys	Lys	Leu	Ser	Leu	Val	Phe	Phe		
		195					200					205					
Gln	Tyr	Cys	Ile	Met	Ala	Asn	Phe	Tyr	Trp	Leu	Leu	Val	Glu	Gly	Leu		
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			245					250						255			
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			260					265					270				
Thr	Asn	Asp	His	Ser	Ile	Pro	Trp	Trp	Val	Ile	Arg	Met	Pro	Ile	Leu		
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Ile	Ser	Ile	Val	Val	Asn	Phe	Ala	Leu	Phe	Ile	Ser	Ile	Val	Arg	Ile		
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Gln	Tyr	Lys	Arg	Leu	Ala	Lys	Ser	Thr	Leu	Leu	Leu	Ile	Pro	Leu	Phe		
				325					330					335			

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370 375 380

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385 390 395 400

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85 90 95

Trp Ser Glu Thr Phe Pro Asp Phe Ile Asp Ala Cys Gly Tyr Asn Asp
100 105 110

Pro Glu Asp Glu Ser Lys Ile Thr Phe Tyr Ile Leu Val Lys Ala Ile
115 120 125

Tyr Thr Leu Gly Tyr Ser Val Ser Leu Met Ser Leu Thr Thr Gly Ser
130 135 140

Ile Ile Ile Cys Leu Phe Arg Lys Leu His Cys Thr Arg Asn Tyr Ile
145 150 155 160

His Leu Asn Leu Phe Leu Ser Phe Met Leu Arg Ala Ile Ser Val Leu
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 Val Val Ala Val Leu Tyr Cys Phe Leu Asn Ser Glu Val Gln Cys Glu
 370 375 380
 Leu Lys Arg Arg Trp Arg Gly Leu Cys Leu Thr Gln Pro Gly Ser Arg
 385 390 395 400
 Asp Tyr Arg Leu His Ser Trp Ser Met Ser Arg Asn Gly Ser Glu Ser
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 Glu Thr Ser Val Ile
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			20					25					30			
Glu	Glu	Thr	Lys	Cys	Ala	Glu	Leu	Leu	Ser	Ser	Gln	Met	Glu	Asn	His	
		35					40					45				
Arg	Ala	Cys	Ser	Gly	Val	Trp	Asp	Asn	Ile	Thr	Cys	Trp	Arg	Pro	Ala	
	50					55					60					
Asp	Ile	Gly	Glu	Thr	Val	Thr	Val	Pro	Cys	Pro	Lys	Val	Phe	Ser	Asn	
65					70					75					80	
Phe	Tyr	Ser	Arg	Pro	Gly	Asn	Ile	Ser	Lys	Asn	Cys	Thr	Ser	Asp	Gly	
				85					90					95		
Trp	Ser	Glu	Thr	Phe	Pro	Asp	Phe	Ile	Asp	Ala	Cys	Gly	Tyr	Asn	Asp	
			100					105					110			
Pro	Glu	Asp	Glu	Ser	Lys	Ile	Thr	Phe	Tyr	Ile	Leu	Val	Lys	Ala	Ile	
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Tyr	Thr	Leu	Gly	Tyr	Ser	Val	Ser	Leu	Met	Ser	Leu	Thr	Thr	Gly	Ser	
		130				135						140				
Ile	Ile	Ile	Cys	Leu	Phe	Arg	Lys	Leu	His	Cys	Thr	Arg	Asn	Tyr	Ile	
145					150					155					160	
His	Leu	Asn	Leu	Phe	Leu	Ser	Phe	Met	Leu	Arg	Ala	Ile	Ser	Val	Leu	
				165					170					175		
Val	Lys	Asp	Ser	Val	Leu	Tyr	Ser	Ser	Ser	Gly	Thr	Leu	Arg	Cys	His	
			180					185					190			
Asp	Gln	Pro	Gly	Ser	Trp	Val	Gly	Cys	Lys	Leu	Ser	Leu	Val	Phe	Phe	
		195					200					205				
Gln	Tyr	Cys	Ile	Met	Ala	Asn	Phe	Tyr	Trp	Leu	Leu	Val	Glu	Gly	Leu	
	210					215						220				
Tyr	Leu	His	Thr	Leu	Leu	Val	Ala	Ile	Leu	Pro	Pro	Ser	Arg	Cys	Phe	
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Leu	Ala	Tyr	Leu	Leu	Ile	Gly	Trp	Gly	Ile	Pro	Ser	Val	Cys	Ile	Gly	
				245					250					255		
Ala	Trp	Ile	Ala	Thr	Arg	Leu	Ser	Leu	Glu	Asp	Thr	Gly	Cys	Trp	Asp	
			260					265					270			
Thr	Asn	Asp	His	Ser	Ile	Pro	Trp	Trp	Val	Ile	Arg	Met	Pro	Ile	Leu	

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 115 120 125
 Tyr Thr Leu Gly Tyr Ser Val Ser Leu Met Ser Leu Thr Thr Gly Ser
 130 135 140
 Ile Ile Ile Cys Leu Phe Arg Lys Leu His Cys Thr Arg Asn Tyr Ile
 145 150 155 160
 His Leu Asn Leu Phe Leu Ser Phe Met Leu Arg Ala Ile Ser Val Leu
 165 170 175
 Val Lys Asp Ser Val Leu Tyr Ser Ser Ser Gly Thr Leu Arg Cys His
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 Asp Gln Pro Gly Ser Trp Val Gly Cys Lys Leu Ser Leu Val Phe Phe
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 Gln Tyr Cys Ile Met Ala Asn Phe Tyr Trp Leu Leu Val Glu Gly Leu
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 Tyr Leu His Thr Leu Leu Val Ala Ile Leu Pro Pro Ser Arg Cys Phe
 225 230 235 240
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 245 250 255
 Ala Trp Ile Ala Thr Arg Leu Ser Leu Glu Asp Thr Gly Cys Trp Asp
 260 265 270
 Thr Asn Asp His Ser Ile Pro Trp Trp Val Ile Arg Met Pro Ile Leu
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 Ile Ser Ile Val Val Asn Phe Ala Leu Phe Ile Ser Ile Val Arg Ile
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 Leu Leu Gln Lys Leu Thr Ser Pro Asp Val Gly Gly Asn Asp Gln Ser
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 Gln Tyr Lys Arg Leu Ala Lys Ser Thr Leu Leu Leu Ile Pro Leu Phe
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 Gly Val His Tyr Met Val Phe Ala Ala Phe Pro Ile Gly Ile Ser Ser
 340 345 350
 Thr Tyr Gln Ile Leu Phe Glu Leu Cys Val Gly Ser Phe Gln Gly Leu
 355 360 365
 Val Val Ala Val Leu Tyr Cys Phe Leu Asn Ser Glu Val Gln Cys Glu
 370 375 380
 Leu Lys Arg Arg Trp Arg Gly Leu Cys Leu Thr Gln Pro Gly Ser Arg
 385 390 395 400

METHODS FOR IDENTIFYING COMPOUNDS FOR REGULATING MUSCLE MASS OR FUNCTION USING VASOACTIVE INTESTINAL PEPTIDE RECEPTORS

Robert J. Isfort

Russell J. Sheldon

TECHNICAL FIELD

The present invention relates to methods of identifying candidate compounds for regulating skeletal muscle mass or function, regulating the activity or expression of a vasoactive intestinal peptide receptors (VPAC) or regulating expression of vasoactive intestinal peptide (VIP) or VIP analogs. The invention also relates to methods for the treatment of skeletal muscle atrophy or methods for inducing skeletal muscle hypertrophy utilizing VPAC receptors as the target for intervention.

SEQUENCE LISTING DESCRIPTION

Each of the VPAC receptor protein sequences included in the sequence listing, along with the corresponding Genbank accession number and animal species from which it is cloned, is shown in Table I.

TABLE I

VPAC Receptor subtype	SEQ ID NO:	SPECIES	CORRESPONDING GENBANK ACCESSION NOS.
VPAC ₁ Receptor	1	human	L13288, U11087
VPAC ₁ Receptor	2	human	X75299
VPAC ₁ Receptor	3	Rattus norvegicus	M86835
VPAC ₁ Receptor	4	Mus musculus	NM_011703
VPAC ₁ Receptor	5	Sus scrofa	U49434
VPAC ₁ Receptor	6	Rana ridibunda	AF100644
VPAC ₁ Receptor	7	Porcine	I28734
VPAC ₁ Receptor	8	Rattus sp	E05551
VPAC ₁ Receptor	9	Carassius auratus	US6391
VPAC ₂ Receptor	10	human	X95097, Y18423
VPAC ₂ Receptor	11	human	L40764, L36566
VPAC ₂ Receptor	12	human	U18810
VPAC ₂ Receptor	13	Mus musculus	D28132
VPAC ₂ Receptor	14	Rattus norvegicus	Z25885
VPAC ₂ Receptor	15	Rattus norvegicus	U09631
VPAC ₂ Receptor	16	Rat	A43808

BACKGROUND

VPAC and ligands

Vasoactive intestinal peptide (VIP) and its functionally and structurally related analogs (VIP analogs), are known to have many physiological functions including smooth muscle relaxation (bronchodilation, intestinal motility), regulation of microvascular tone (vasodepression) and permeability, regulation of mucus secretion, modulation of various immune functions (anti-inflammation, immune cell protection), neurological effects (memory improvement, hypnogenesis, food intake, circadian rhythm control, sexual behavior), maintenance of salivary gland function, developmental growth regulation and stimulation of hormone secretion (prolactin, growth hormone, insulin). VIP and VIP analogs mediate their effects through vasoactive intestinal peptide receptors via both neuronal (as putative neurotransmitters) and neuroendocrine pathways. There are two VPAC receptors identified to date (VPAC₁ and VPAC₂). The VPAC₁ receptor has been cloned from human, mouse (*Mus musculus*), rat (*Rattus norvegicus* and *Rattus sp.*), pig (*Sus scrofa*), frog (*Rana ridibunda*), goldfish (*Carassius auratus*), and turkey (*Meleagris gallopavo*). The VPAC₂ receptor has been cloned from human, mouse (*Mus musculus*), and rat (*Rattus norvegicus*).

VPAC₁ and VPAC₂ receptors are classified in the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor family based on sequence homology to other members of the PACAP family. Receptors in the PACAP family are further subdivided into two subclasses based on ligand affinity. The PACAP type I receptors have a much greater affinity for PACAP than for VIP, while the PACAP type II receptors have an approximately equal affinity for PACAP and VIP. Because VPAC₁ and VPAC₂ receptors have similar affinities for PACAP and VIP, these receptors are classified as PACAP type II receptors. Selective agonists and antagonists can differentiate VPAC₁ and VPAC₂ receptors from each other, both molecularly and pharmacologically, as well as from the PACAP type I receptors. These agonist and antagonists have been useful in matching biological activity to a particular VPAC receptor subclass.

VPAC₁ and VPAC₂ receptors both belong to the G-protein coupled receptor (GPCR) class. The specificity of coupling of VPAC₁ and VPAC₂ receptors to a particular G-protein, appears to depend upon the tissue examined. In tissues such as muscle, agonist activation of VPAC₁ or VPAC₂ receptors leads to G_{os} activation of adenylate cyclase. Adenylate cyclase catalyzes the formation of cAMP which in turn has multiple effects including the activation of protein kinase A, intracellular calcium release and mitogen-activated protein kinase (MAP kinase) activation. In

other studies, the enhancement of intracellular inositol triphosphate synthesis after agonist activation of VPAC receptors suggests VPAC receptor coupling to either $G_{\alpha i}$ or $G_{\alpha q}$.

Expression of VPAC₁ and VPAC₂ receptors is tissue specific and the pattern of expression of each receptor differs. In humans, the VPAC₁ receptor has been shown to be expressed in brain, adipose, liver, and heart, while the VPAC₂ receptor has been shown to be expressed in lung, pancreas, brain, kidney, skeletal muscle, stomach, heart, and placenta. In the rat, expression of the VPAC₁ receptor has been found in the pineal gland, small intestine, liver, spleen, pancreas, lung, aorta, vas deferens and brain, while expression of the VPAC₂ receptor has been shown in the stomach, intestine, skeletal muscle, spleen, pancreas, thymus, adrenal gland, heart, lung, aorta, brain, pituitary, and olfactory bulb.

Skeletal Muscle Atrophy and Hypertrophy

Skeletal muscle is a plastic tissue which readily adapts to changes in either physiological demand for work or metabolic need. Hypertrophy refers to an increase in skeletal muscle mass while skeletal muscle atrophy refers to a decrease in skeletal muscle mass. Acute skeletal muscle atrophy is traceable to a variety of causes including, but not limited to: disuse due to surgery, bed rest, or broken bones; denervation/nerve damage due to spinal cord injury, autoimmune disease, or infectious disease; glucocorticoid use for unrelated conditions; sepsis due to infection or other causes; nutrient limitation due to illness or starvation; and space travel. Skeletal muscle atrophy occurs through normal biological processes, however, in certain medical situations this normal biological process results in a debilitating level of muscle atrophy. For example, acute skeletal muscle atrophy presents a significant limitation in the rehabilitation of patients from immobilizations, including, but not limited to, those accompanying an orthopedic procedure. In such cases, the rehabilitation period required to reverse the skeletal muscle atrophy is often far longer than the period of time required to repair the original injury. Such acute disuse atrophy is a particular problem in the elderly, who may already suffer from substantial age-related deficits in muscle function and mass, because such atrophy can lead to permanent disability and premature mortality.

Skeletal muscle atrophy can also result from chronic conditions such as cancer cachexia, chronic inflammation, AIDS cachexia, chronic obstructive pulmonary disease (COPD), congestive heart failure, genetic disorders, e.g. muscular dystrophies, neurodegenerative diseases and sarcopenia (age associated muscle loss). In these chronic conditions, skeletal muscle atrophy can lead to premature loss of mobility, thereby adding to the disease-related morbidity.

Little is known regarding the molecular processes which control atrophy or hypertrophy of skeletal muscle. While the initiating trigger of the skeletal muscle atrophy is different for the various atrophy initiating events, several common biochemical changes occur in the affected skeletal muscle fiber, including a decrease in protein synthesis and an increase in protein degradation and changes in both contractile and metabolic enzyme protein isoforms characteristic of a slow (highly oxidative metabolism/slow contractile protein isoforms) to fast (highly glycolytic metabolism/fast contractile protein isoforms) fiber switch. Additional changes in skeletal muscle which occur include the loss of vasculature and remodeling of the extracellular matrix. Both fast and slow twitch muscle demonstrate atrophy under the appropriate conditions, with the relative muscle loss depending on the specific atrophy stimuli or condition. Importantly, all these changes are coordinately regulated and are switched on or off depending on changes in physiological and metabolic need.

The processes by which atrophy and hypertrophy occur are conserved across mammalian species. Multiple studies have demonstrated that the same basic molecular, cellular, and physiological processes occur during atrophy in both rodents and humans. Thus, rodent models of skeletal muscle atrophy have been successfully utilized to understand and predict human atrophy responses. For example, atrophy induced by a variety of means in both rodents and humans results in similar changes in muscle anatomy, cross-sectional area, function, fiber type switching, contractile protein expression, and histology. In addition, several agents have been demonstrated to regulate skeletal muscle atrophy in both rodents and in humans. These agents include anabolic steroids, growth hormone, insulin-like growth factor I, and beta adrenergic agonists. Together, these data demonstrate that skeletal muscle atrophy results from common mechanisms in both rodents and humans.

While some agents have been shown to regulate skeletal muscle atrophy and are approved for use in humans for this indication, these agents have undesirable side effects such as hypertrophy of cardiac muscle, neoplasia, hirsutism, androgenization of females, increased morbidity and mortality, liver damage, hypoglycemia, musculoskeletal pain, increased tissue turgor, tachycardia, and edema (54th Edition of the Physicians Desk Reference, 2000). Currently, there are no highly effective and selective treatments for either acute or chronic skeletal muscle atrophy. Thus, there is a need to identify other therapeutic agents which regulate skeletal muscle atrophy.

One problem associated with identification of compounds for use in the treatment of skeletal muscle atrophy has been the lack of good screening methods for the identification of such compounds. Applicants have now found that VPAC₁ and VPAC₂ receptors are involved in the

regulation of skeletal muscle mass or function and that agonists of VPAC₁ and VPAC₂ receptors are able to block skeletal muscle atrophy and/or induce hypertrophy of skeletal muscle.

SUMMARY OF THE INVENTION

The present invention relates to the use of VPAC receptors to identify candidate
5 compounds that are potentially useful in the treatment of skeletal muscle atrophy and/or useful to induce skeletal muscle hypertrophy. In particular, the invention provides *in vitro* methods for identifying candidate compounds for regulating skeletal muscle mass or function. In one embodiment of the invention the method comprises: contacting a test compound with a VPAC
10 receptor, and determining whether the test compound binds to the VPAC receptor, wherein test compounds that bind to the VPAC receptor are identified as candidate compounds for regulating skeletal muscle mass or function. In another embodiment of the invention the method comprises: contacting a test compound with a cell expressing a VPAC receptor, and determining whether the test compound activates the VPAC receptor, wherein test compounds that activate the VPAC
15 receptor are identified as candidate compounds for regulating skeletal muscle mass or function. In yet another embodiment of the invention the method further comprises generating a list of candidate compounds.

In another aspect, the present invention relates to the use of VPAC receptors to identify candidate therapeutic compounds which regulate skeletal muscle mass or function *in vivo*. In particular, the invention provides a method comprising: contacting a test compound with a VPAC
20 receptor, determining whether the test compound binds to the VPAC receptor, administering a test compound determined in the previous step to bind to the VPAC receptor, or previously known to bind to the VPAC receptor, to a non-human animal and determining whether the test compound regulates skeletal muscle mass or muscle function in the treated animal. Those test compounds that regulate skeletal muscle mass or function are identified as candidate therapeutic compounds for
25 regulating skeletal muscle mass or function *in vivo*. In another embodiment of the invention the method comprises: contacting a test compound with a cell expressing VPAC receptors, determining whether the test compound activates the VPAC receptor, administering a test compound determined in the previous step to activate the VPAC receptor, or previously known to activate the VPAC
30 receptor, to a non-human animal and determining whether the test compound regulates skeletal muscle mass or muscle function in the treated animal. Those test compounds that regulate skeletal muscle mass or function *in vivo* are identified as candidate therapeutic compounds for regulating skeletal muscle mass or function *in vivo*.

The invention further provides methods for identifying candidate compounds that prolong or augment the activation of VPAC receptors or of a VPAC receptor signal transduction pathway. These methods comprise, (i) contacting a cell which expresses functional VPAC receptors with a VPAC receptor agonist at a concentration of agonist and for a period of agonist-receptor exposure sufficient to allow desensitization of the receptor; (ii) contacting the cells with a test compound; and (iii) determining the level of activation of the VPAC receptor. In the above-described embodiment of the invention, step (ii) may be performed before or after step (i). In a particular embodiment, the present invention relates to a method of determining whether those candidate compounds that prolong or augment the agonist-induced activation of VPAC receptors or a VPAC receptor signal transduction pathway, can be used to regulate skeletal muscle mass or function *in vivo* by administering a candidate compound, alone or in conjunction with a VPAC receptor agonist, to a non-human animal and determining whether the candidate compound regulates skeletal muscle mass or function in the treated animal. Those candidate compounds that regulate skeletal muscle mass or function *in vivo* are identified as candidate therapeutic compounds for regulating skeletal muscle mass or function *in vivo*.

The invention further provides methods for identifying candidate compounds that increase VPAC receptor expression comprising contacting a test compound with a cell or cell lysate containing a reporter gene operatively associated with a VPAC receptor gene regulatory element and detecting expression of the reporter gene. Test compounds that increase expression of the reporter gene are identified as candidate compounds for increasing VPAC receptor expression. In a particular embodiment, the present invention relates to a method of determining whether those candidate compounds which increase VPAC receptor expression can be used to regulate skeletal muscle mass or function *in vivo* by administering a candidate compound to a non-human animal and determining whether the candidate compound regulates skeletal muscle mass or function in the treated animals. Those candidate compounds that regulate skeletal muscle mass or function *in vivo* are identified as candidate therapeutic compounds for regulating skeletal muscle mass or function *in vivo*.

In another embodiment the invention provides for antibodies specific for VPAC receptors. In particular the invention provides for chimeric or human antibodies specific for VPAC receptors.

The invention additionally provides methods for identifying compounds that increase VIP or VIP analog expression which include the steps of contacting a test compound with a cell or cell lysate containing a reporter gene operatively associated with a VIP or VIP analog gene regulatory element and detecting expression of the reporter gene. Test compounds that increase reporter gene

expression are identified as candidate compounds which increase VIP or VIP analog expression. In a particular embodiment, the present invention relates to a method of determining whether those candidate compounds which increase VIP or VIP analog expression regulate skeletal muscle mass or function *in vivo* by administering a candidate compound to a non-human animal and determining whether the candidate compound regulates skeletal muscle mass or function in the treated animal. Those candidate compounds that regulate skeletal muscle mass or function *in vivo* are identified as candidate therapeutic compounds for regulating skeletal muscle mass or function *in vivo*.

The present invention also relates to use of a VPAC receptor as a therapeutic target to increase skeletal muscle mass or function. This use includes, the use of a VPAC receptor agonist, a compound that prolongs or augments the activation of VPAC receptors or the activation of a VPAC receptor signal transduction pathway, an expression vector encoding a functional VPAC receptor, an expression vector encoding a constitutively active VPAC receptor, a compound that increases expression of VPAC receptors, a compound that increases expression of VIP or a compound that increases expression of a VIP analog to increase skeletal muscle mass or function and/or to treat skeletal muscle atrophy. In particular, the invention provides a method for increasing skeletal muscle mass or function in a subject in which such an increase is desirable, comprising: identifying a subject in which an increase in skeletal muscle mass or function is desirable and administering to the subject a safe and effective amount of a VPAC receptor agonist, a compound that prolongs or augments the activation of VPAC receptors or the activation of a VPAC receptor signal transduction pathway, an expression vector encoding a functional VPAC receptor, an expression vector encoding a constitutively active VPAC receptor, a compound that increases expression of VPAC receptors, a compound that increases expression of VIP or a compound that increases expression of a VIP analog. In another embodiment, the invention provides methods of treating skeletal muscle atrophy, in a subject in need of such treatment, comprising administering to the subject an effective amount of a VPAC receptor agonist, a compound that prolongs or augments the activation of VPAC receptors or the activation of a VPAC receptor signal transduction pathway, an expression vector encoding a functional VPAC receptor, an expression vector encoding a constitutively active VPAC receptor, a compound that increases expression of VPAC receptors, a compound that increases expression of VIP or a compound that increases expression of VIP analog.

The present invention also relates to the use of a compound that prolongs or augments the activation of VPAC receptors, or of a VPAC receptor signal transduction pathway, to increase skeletal muscle mass or function and/or to treat skeletal muscle atrophy. In particular, the

invention provides methods of treating skeletal muscle atrophy, in a subject in need of such treatment, comprising administering, alone or in conjunction with a VPAC receptor agonist, a safe and effective amount of a compound that prolongs or augments the activation of VPAC receptors, or of a VPAC receptor signal transduction pathway.

5

DESCRIPTION OF THE FIGURES

Abbreviations used in figures 1-5:

VPAC ₁ R agonist	-	[K ¹⁵ , R ¹⁶ , L ²⁷ VIP(1-7), GRF(8-27)-NH ₂]
VPAC ₂ R agonist	-	Ro 25-1553
PACAP-38	-	VPAC ₁ / VPAC ₂ receptor non-selective agonist
Saline	-	physiological saline (vehicle control)
T	-	theophylline (30 mg/kg - administered 2X daily)
g	-	gram
*	-	statistically significant response versus saline

FIG. 1A and 1B demonstrate the anti-atrophy effects of VPAC₁ and VPAC₂ receptor agonists (administered subcutaneously, 2X daily) in the mouse denervation atrophy model. FIG 1A shows the anti-atrophy effects of VPAC₁ and VPAC₂ receptor selective agonists in denervated tibialis anterior muscle. FIG 1B shows the anti-atrophy effects of VPAC₁ and VPAC₂ receptor selective agonists in denervated medial gastrocnemius muscle. Legend for the X-axis: A: Saline, B: VPAC₁R agonist (0.1 mg/kg) + T, C: VPAC₁R agonist (0.3 mg/kg) + T, D: VPAC₂R agonist (0.1 mg/kg) + T, E: VPAC₂R agonist (0.3 mg/kg) + T, F: PACAP-38 (0.1 mg/kg) + T, G: PACAP-38 (0.3 mg/kg) + T.

FIG. 2A and 2B demonstrate the anti-atrophy and hypertrophy-inducing effects of VPAC₁ and VPAC₂ receptor selective agonists (administered continuously by osmotic minipump) in the mouse denervation atrophy model. FIG 2A shows the anti-atrophy and hypertrophy-inducing effects of the VPAC₂ receptor selective agonist in denervated and normal tibialis anterior muscle. FIG 2B shows the hypertrophy-inducing effects of the VPAC₂ receptor selective agonist in normal medial gastrocnemius muscle. Legend for the X-axis: A: water infused, B: VPAC₁R agonist (0.3 mg/kg), C: VPAC₁R agonist (1 mg/kg), D: VPAC₁R agonist (3 mg/kg), E: VPAC₂R agonist (0.3 mg/kg), F: VPAC₂R agonist (1 mg/kg), G: VPAC₂R agonist (3 mg/kg), H: PACAP-38 (0.3 mg/kg), I: PACAP-38 (1 mg/kg), PACAP-38 (3 mg/kg).

FIG. 3A and 3B demonstrate the anti-atrophy effects of VPAC₁ and VPAC₂ receptor selective agonists (administered subcutaneously, 2X daily) in the mouse glucocorticoid (dexamethasone)-induced atrophy model. FIG 3A shows the anti-atrophy effects of VPAC₁ and VPAC₂ receptor selective agonists in glucocorticoid-induced atrophied tibialis anterior muscle. FIG 3B shows the anti-atrophy effects of VPAC₁ and VPAC₂ receptor selective agonists in glucocorticoid-induced atrophied medial gastrocnemius muscle. Legend for the X-axis: A: Saline + Dexamethasone (1.2 mg/kg/day included in the drinking water), B: PACAP-38 (0.1 mg/kg) + Dexamethasone + T, C: PACAP-38 (0.3 mg/kg) + Dexamethasone + T, D: VPAC₁R agonist (0.1 mg/kg) + Dexamethasone + T, E: VPAC₁R agonist (0.3 mg/kg) + Dexamethasone + T, F: VPAC₂R agonist (0.1 mg/kg) + Dexamethasone + T, G: VPAC₂R agonist (0.3 mg/kg) + Dexamethasone + T.

FIG. 4A and 4B demonstrate the anti-atrophy and hypertrophy-inducing effects of a VPAC₂ receptor selective agonist (administered subcutaneously, 2X daily) in the mouse disuse (casting) atrophy model. FIG 4A shows the anti-atrophy effects of the VPAC₂ receptor selective agonist, Ro 25-1553, in casting-induced atrophied tibialis anterior muscle. FIG 4B shows the hypertrophy-inducing effects of VPAC₂ receptor selective agonist, Ro 25-1553 in normal medial gastrocnemius muscle. Legend for the X-axis: A: Saline, B: VPAC₂R agonist (0.03 mg/kg) + T, C: VPAC₂R agonist (0.1 mg/kg) + T, D: VPAC₂R agonist (0.3 mg/kg) + T.

FIG. 5A and 5B demonstrate the anti-atrophy effects of a VPAC₂ receptor selective agonist (administered subcutaneously, 2X daily) in the rat denervation atrophy model. FIG 5A shows the anti-atrophy effects of the VPAC₂ receptor selective agonist, Ro 25-1553, in denervation-induced atrophied tibialis anterior muscle. FIG 5B shows the anti-atrophy effects of VPAC₂R agonist, Ro 25-1553 in denervation-induced atrophied extensor digitorum longus (EDL) muscle. Legend for the X-axis: A: Saline, B: VPAC₂R agonist (0.03 mg/kg) + T, C: VPAC₂R agonist (0.1 mg/kg) + T, D: VPAC₂R agonist (0.3 mg/kg) + T.

DETAILED DESCRIPTION OF THE INVENTION

I. Terms and Definitions:

The following is a list of definitions for terms used herein.

“Agonist” means any compound that activates a receptor.

“Allelic variant” means a variant form of a given gene or gene product. One of skill in the art recognizes that a large number of genes are present in two or more allelic forms in a population and some genes have numerous alleles.

“Antibody”, in its various grammatical forms, means immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds an antigen. “Purified antibody” means an antibody which has been partially or completely separated from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 60% antibody, more preferably at least 75% antibody, more preferably at least 90% antibody, and most preferably at least 99%, by dry weight, antibody.

“Binding affinity” means the propensity for a ligand to interact with a receptor and is inversely related to the dissociation constant for a specific VIP ligand-VPAC interaction. The dissociation constant can be measured directly via standard saturation, competition, or kinetics binding techniques or indirectly via pharmacological techniques involving functional assays and endpoints.

“Chimeric antibody” means an antibody that contains structural elements from two or more different antibody molecules, i.e. from different animal species. Chimeric antibodies include, but are not limited to, antibodies known as “humanized antibodies” which include, but are not limited to, chimeric antibodies generated by the technique known as complementarity determining region grafting.

“Fusion” means two or more DNA coding sequences operably associated so as to encode one hybrid protein. A “fusion protein” therefore refers to such a hybrid protein.

“Inhibit” means to partially or completely block a particular process or activity. For example, a compound inhibits skeletal muscle atrophy if it either completely or partially prevents muscle atrophy.

As used herein, two DNA sequences are said to be “operably associated” if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of a promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. For example, a coding sequence and regulatory sequences are operably associated when they are covalently linked in such a way as to place the transcription of the coding sequence under the influence or control of the regulatory sequences. Thus, a promoter region is operably associated with a coding sequence when the promoter region is capable of effecting transcription of

that DNA sequence such that the resulting transcript is capable of being translated into the desired protein or polypeptide.

“PACAP” means pituitary adenylate cyclase-activating polypeptide.

“Percent identity” means the percentage of nucleotides or amino acids that two sequences have in common as calculated as follows. To calculate the percent identity for a specific sequence (the query), the relevant part of the query sequence is compared to a reference sequence using the BestFit comparison computer program, Wisconsin Package, Ver. 10.1, available from the Genetics Computer Group, Inc. This program uses the algorithm of Smith and Waterman, *Advances in Applied Mathematics*, Issue 2: 482-489 (1981). Percent identity is calculated with the following default parameters for the BestFit program: the scoring matrix is blosum62.cmp, the gap creation penalty is 8 and the Gap extension penalty is 2. When comparing a sequence to the reference sequence, the relevant part of the query sequence is that which is derived from a VPAC sequence. For example, where the query is a VPAC/purification tag fusion protein, only the VPAC polypeptide portion of the sequence is aligned to calculate the percent identity score.

“Polypeptide” means any chain of amino acids, regardless of length or post-translational modification (e.g., phosphorylation or glycosylation).

“Promoter” means a DNA sequence which controls the initiation of transcription and the rate of transcription from a gene or coding region.

“Prophylactic treatment” means preventive treatment of a subject, not currently exhibiting signs of skeletal muscle atrophy, in order to completely or partially block the occurrence of skeletal muscle atrophy. One of skill in the art would recognize that certain individuals are at risk for skeletal muscle atrophy as discussed in the background section herein. Furthermore, one of skill in the art would recognize that if the biochemical changes leading to skeletal muscle atrophy are appropriately regulated, that the occurrence of atrophy would be prevented or reduced in at-risk individuals.

“Regulate” in all its grammatical forms, means to increase, decrease or maintain, e.g. to regulate skeletal muscle mass or function means to increase, decrease or maintain the level of skeletal muscle mass or function.

“Regulation of skeletal muscle mass or function” includes regulation of skeletal muscle mass, skeletal muscle function or both.

“Regulatory element” means a DNA sequence that is capable of controlling the level of transcription from an operably associated DNA sequence. Included within this definition of

regulatory element are promoters and enhancers. E.g., a VPAC receptor gene regulatory element is a DNA sequence capable of controlling the level of transcription from the VPAC receptor gene.

“Reporter gene” means a coding sequence whose product can be detected, preferably quantitatively, wherein the reporter gene is operably associated with a heterologous promoter or enhancer element which is responsive to a signal which is to be measured. The promoter or enhancer element in this context is referred to herein as a “responsive element”.

“Selective agonist” means that the agonist has significantly greater activity toward a certain receptor(s) compared with other receptors, not that it is completely inactive with regard to other receptors.

“Skeletal muscle hypertrophy” means an increase in skeletal muscle mass or skeletal muscle function or both.

“Skeletal muscle atrophy” means the same as “muscle wasting” and means a decrease in skeletal muscle mass or skeletal muscle function or both.

“Splice variant” means a mRNA or protein which results from alternative exon usage. One of skill in the art recognizes that, depending on cell type, or even within a single cell type, a mRNA may be expressed in a slightly different form, as a splice variant, and thus the translated protein will be different depending upon the mRNA that is expressed.

A “therapeutically effective amount” of a substance is an amount capable of producing a medically desirable result in a treated subject, e.g., decreases skeletal muscle atrophy, increases skeletal muscle mass or increases skeletal muscle function, with an acceptable benefit: risk ratio; in a human or non-human mammal.

“Therapeutic treatment” means treatment of a subject in which an increase in muscle mass or muscle function is desirable. For example, treatment of a subject currently exhibiting signs of skeletal muscle atrophy in order to partially or completely reverse the skeletal muscle atrophy that has occurred or to completely or partially block the occurrence of further skeletal muscle atrophy would be therapeutic treatment of that subject. The term “therapeutic treatment” also includes, for example, treatment of a subject not exhibiting signs of skeletal muscle atrophy to induce skeletal muscle hypertrophy, e.g. treatment of a livestock animal to increase muscle mass

The term “treatment” means prophylactic or therapeutic treatment.

“VIP” means vasoactive intestinal peptide.

“VIP analog” means polypeptides which act as ligands of VPAC receptors. Preferred VIP analogs are PACAP-27, PACAP-38, helodermin, peptide histidine isoleucine amide (PHI), peptide histidine methionine amide, peptide histidine valine amide (PHV), growth hormone releasing

hormone, secretin, glucagons, (Arg15, Arg21) VIP, [(Arg15,20,21Leu17)-VIP-Gly-Lys-Arg-NH₂], [K¹⁵, R¹⁶, L²⁷,VIP(1-7), GRF(8-27)-NH₂], multimeric VIP fusion proteins, Ro25-1553, Ro 25-1392 or PACAP(6-38). More preferred VIP analogs are VIP, PACAP-27, PACAP-38, PHI, PHV, Ro25-1553, Ro 25-1392 and [K¹⁵, R¹⁶, L²⁷,VIP(1-7), GRF(8-27)-NH₂].

5 “VPAC receptor agonist” means a compound or molecule which has the ability to activate the VPAC₁ or the VPAC₂ receptor, or both. Activation of VPAC receptors can be measured as described hereinafter.

The nomenclature regarding the VPAC receptors used herein follows the convention proposed by Harmar, et al. in *Pharmacological Reviews* (1998) 50(2): 265-270.

10 “VPAC receptor” means the VPAC₁ receptor or VPAC₂ receptor.

“VPAC₁ receptor” means the VPAC₁ receptor from any animal species. The VPAC₁ receptor has previously been referred to as the “VIP receptor”, “VIP₁ receptor” (Lutz *et al.*, *FEBS Lett* (1993) 334:3-8, “VIP/PACAP Type II Receptor” (Ciccarelli *et al.*, *Regul Pept* (1994) 54:397-407, “HIVR” or “human intestinal VIP receptor” (Couvineau, A. et. al., *Biochem. Biophys. Res. Commun.*(1994) 200(2), 769-776, and “PVR2” (Rawlings *et al.*, *Endocrinology* (1995) 136:2088-2098.

The definition of VPAC₁ receptor includes, but is not limited to, those receptors for which the cDNA or genomic sequence encoding the receptor has been deposited in a sequence database. These sequences include Accession Nos.: L13288, X75299, M86835, NM_011703, U49434, 20 AF100644, I28734, U31991 (partial cDNA sequence), U56391, E05551 and 806702. The protein sequence of these VPAC₁ receptors is obtained by translation of the coding region of these DNA sequences and is generally available as part of the database entry.

“VPAC₂ receptor” means the VPAC₂ receptor from any animal species. The VPAC₂ receptor has also been referred to as “VIP2” (Lutz *et al.*, 1993), “PACAPR-3” (Inagaki *et al.*, 25 (1994) *Proc Natl Academy Sci USA* 91:2679-2683 and “PVR3” (Rawlings *et al.*, (1995) *Endocrinology* 136:2088-2098.

The definition of VPAC₂ receptor includes, but is not limited to, those receptors for which the DNA sequence encoding the receptor has been deposited in a sequence database. These sequences include Accession Nos.: X95097, L40764, L36566, Y18423, U18810, D28132, 30 Z25885, U09631 and A43808. The protein sequence of these VPAC₂ receptors is obtained by translation of the coding region of these DNA sequences and is generally available as part of the database entry.

The term "VPAC receptor" includes truncated and/or mutated proteins wherein regions of the receptor molecule not required for ligand binding or signaling have been deleted or modified. For example one of skill in the art will recognize that a VPAC receptor with one or more conservative changes in the primary amino acid sequence would be useful in the present invention.

5 It is known in the art that substitution of certain amino acids with different amino acids with similar structure or properties (conservative substitutions) can result in a silent change, i.e. a change that does not significantly alter function. Conservative substitutes are well known in the art. For example, it is known that GPCRs can tolerate substitutions of amino acid residues in the transmembrane alpha-helices, which are oriented toward lipid, with other hydrophobic amino acids, and remain functional. VPAC₁ receptors differing from a naturally occurring sequence by truncations and/or mutations such as conservative amino acid substitutions are included in the definition of VPAC₁ receptor. VPAC₂ receptors differing from a naturally occurring sequence by truncations and/or mutations such as conservative amino acid substitutions are included in the definition of VPAC₂ receptor.

15 One of skill in the art would also recognize that VPAC receptors from a species other than those listed above, particularly mammalian species, would be useful in the present invention. One of skill in the art would further recognize that by using probes from the known VPAC species' sequences, cDNA or genomic sequences homologous to the known sequence could be obtained from the same or alternate species by known cloning methods. Such VPAC₁ receptors are included in the definition of VPAC₁ and such VPAC₂ receptors are included in the definition of VPAC₂.

20 In addition, one of skill in the art would recognize that allelic variants or splice variants of VPAC receptors might be present in a particular species and that these variants would have utility in the present invention. Such VPAC₁ receptor variants are included in the definition of VPAC₁ and such VPAC₂ receptor variants are included in the definition of VPAC₂.

25 Fusions of a VPAC₁ or VPAC₂ receptor polypeptide, or a VPAC₁ or VPAC₂ receptor polypeptide fragment to a non-VPAC polypeptide are referred to as VPAC receptor fusion proteins. Using known methods, one of skill in the art would be able to make fusion proteins of a VPAC₁ receptor or a VPAC₂ receptor that, while different from native VPAC₁ and VPAC₂ receptors, would remain useful in the present invention. VPAC₁ receptor fusion proteins are included within the definition of VPAC₁ receptor and VPAC₂ receptor fusion proteins are included within the definition of VPAC₂ receptor.

30 "Functional VPAC receptors" refers to VPAC receptors that bind VIP or a VIP analog *in vivo* or *in vitro* and are activated as a result of ligand binding.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the arts of protein chemistry, pharmacology, or molecular biology. The methods, materials and examples described herein are not intended to be limiting. Other methods and materials similar or equivalent to those described
5 herein can be used in the practice or testing of the present invention.

II. The Role of VPAC Receptors in Regulation of Skeletal Muscle Mass

One of skill in the art would recognize the utility of the present invention given the information in the prior art and the teachings below. The results herein show that agonists of VPAC receptors induce skeletal muscle hypertrophy and/or inhibit skeletal muscle atrophy induced
10 by denervation, disuse and corticosteroids, thus demonstrating the modulatory role and function of the VPAC receptors in the process of skeletal muscle atrophy. Not wishing to be bound by theory, it is thought that the effect of VPAC receptor agonists on skeletal muscle atrophy and hypertrophy is due to a direct effect on the muscle cells. However, it is also possible that this effect is mediated, in whole, or in part, through a secretagogue effect of the agonist on a non-muscle tissue.

The specific role of VPAC receptors *in vivo* was investigated using pharmacological
15 agents, which are selective agonists for either PACAP receptors, the VAPC₁ receptor or the VPAC₂ receptor, in various models of skeletal muscle atrophy, described hereinafter. Selective agonists used include: PACAP-38 (non-selective for the VAPC₁/VPAC₂/PAC₁ receptors) (Bachem Biosciences Inc., King of Prussia, PA); [K¹⁵, R¹⁶, L²⁷, VIP(1-7), GRF(8-27)-NH₂] (VPAC₁ receptor
20 selective agonist with potency similar to PACAP38) which was synthesized according to the method of Gourlet, Vertongen et al., *Peptides* 18 (1997) 403-408; and Ro 25-1553 (VPAC₂ receptor selective agonist with potency similar to PACAP38) which was purchased from Synpep Inc. (Dublin, CA). Before use, the agents were checked for purity by HPLC and for composition by mass spectrometry. These agents have been well characterized and are described in the
25 scientific literature (Gourlet, Vandermeers et al., *Peptides* 18 (1997) 1539-1545; Gourlet, Vertongen et al., *Peptides* 18 (1997) 403-408; Gozes et al., *Curr. Med. Chem.* 6 (1999) 1019-1034).

Figures 1-5 show the results of experiments demonstrating that administration of agonists for VPAC₁ or VPAC₂ receptors result in statistically significant inhibition of skeletal muscle
30 atrophy and or induction of hypertrophy. VPAC₁ or VPAC₂ receptor agonists administered twice daily in combination with theophylline resulted in inhibition of skeletal muscle atrophy in animal models of skeletal muscle atrophy. Theophylline was added to potentiate the duration and

magnitude of action of the VPAC₁ and VPAC₂ receptor agonists therefore resulting in increased efficacy of these compounds. Theophylline administered by itself in these atrophy models had no effect, demonstrating that the anti-atrophy effect of the VPAC₁ and VPAC₂ receptor agonists in combination with theophylline was due to the effect of the VPAC receptor agonists. Furthermore, continuous dosing of the VPAC₁ and VPAC₂ receptor agonists in the absence of theophylline, via osmotic mini-pump, also resulted in inhibition of skeletal muscle atrophy and or in skeletal muscle hypertrophy.

Specifically, Figure 1 (FIG. 1.) shows the results of a mouse denervation atrophy study utilizing the VPAC₁ receptor selective agonist, [K¹⁵, R¹⁶, L²⁷ VIP(1-7), GRF(8-27)-NH₂] (VPAC1R agonist), the VPAC₂ receptor selective agonist, Ro 25-1553 (VPAC2R agonist), or the VPAC₁ / VPAC₂ receptor nonselective agonist, PACAP-38. Following denervation of the right sciatic nerve, male mice were injected subcutaneously in the midscapular region twice daily, with either the above indicated agents at the doses indicated in Figures 1A and 1B or vehicle control (physiological saline) for nine days. These agonists were co-administered with twice daily intra-peritoneal dosing of the phosphodiesterase inhibitor theophylline (T - 30 mg/kg) (included to increase the magnitude and the duration of action of cAMP signals induced by the VPAC receptor agonists). On day nine, the medial gastrocnemius and tibialis anterior muscles were removed and weighed to determine the degree of atrophy. This experiment demonstrated that the non-specific VPAC receptor agonist, PACAP-38 inhibited atrophy of the denervated tibialis anterior (FIG 1A) and medial gastrocnemius muscles (FIG 1B). The VPAC₁ receptor selective agonist [K¹⁵, R¹⁶, L²⁷ VIP(1-7), GRF(8-27)-NH₂] inhibited atrophy of the denervated medial gastrocnemius muscle (FIG 1B). The VPAC₂ receptor selective agonist Ro 25-1553 inhibited atrophy of the denervated tibialis anterior (FIG 1A) and medial gastrocnemius (FIG 1B) muscles. Statistical significance of the results were determined using ANCOVA (Douglas C. Montgomery, Design and Analysis of Experiments, John Wiley and Sons, New York (2nd ed. 1984)).

Figure 2 (FIG. 2.) shows the results of a mouse denervation atrophy study utilizing the VPAC₁ receptor selective agonist, [K¹⁵, R¹⁶, L²⁷ VIP(1-7), GRF(8-27)-NH₂] (VPAC1R agonist), the VPAC₂ receptor selective agonist, Ro 25-1553 (VPAC2R agonist), or the VPAC₁ / VPAC₂ receptor nonselective agonist, PACAP-38. Following denervation of the right sciatic nerve, male mice were dosed with the above indicated agents or vehicle control (physiological saline) by continuous infusion using an ALZeT osmotic minipump at 5µl/hr until the end of the experimental period. The daily delivered dose of the agents are as indicated on Figures 2A and 2B. Minipump implantation was performed at the same time as the sciatic nerve denervation. On day nine the

medial gastrocnemius and tibialis anterior muscles were removed and weighed to determine the degree of atrophy. The VPAC₂ receptor selective agonist Ro 25-1553 inhibited atrophy of the denervated tibialis anterior (FIG 2A) muscle. In addition, the VPAC₂ receptor selective agonist, Ro 25-1553, induced hypertrophy of the non-denervated (control) tibialis anterior (FIG 2A) and medial gastrocnemius (FIG 2B) muscles. Statistical significance of the results were determined using ANCOVA.

Figure 3 (FIG. 3.) shows the results of a mouse glucocorticoid-induced atrophy study utilizing the VPAC₁ receptor selective agonist, [K¹⁵, R¹⁶, L²⁷ VIP(1-7), GRF(8-27)-NH₂] (VPAC₁R agonist), the VPAC₂ receptor selective agonist, Ro 25-1553 (VPAC₂R agonist), or the VPAC₁ / VPAC₂ receptor nonselective agonist, PACAP-38. Following the addition of the glucocorticoid, dexamethasone to the drinking water (1.2 mg/kg/d), male mice were injected subcutaneously in the midscapular region twice daily, with either the above indicated agents at the doses indicated in Figures 3A and 3B or vehicle control (physiological saline) for nine days. These agonists were co-administered with twice daily intra-peritoneal dosing of the phosphodiesterase inhibitor theophylline (T - 30 mg/kg). Nine days following the initiation of dosing of the VPAC receptor agonists and dexamethasone, the medial gastrocnemius and tibialis anterior muscles were removed and weighed to determine the degree of atrophy. This experiment demonstrates that the non-specific VPAC receptor agonist, PACAP-38, inhibits muscle atrophy of the medial gastrocnemius atrophy induced by glucocorticoid administration (FIG 3B). The VPAC₁ receptor selective agonist [K¹⁵, R¹⁶, L²⁷ VIP(1-7), GRF(8-27)-NH₂] inhibited muscle atrophy of the tibialis anterior induced by glucocorticoid administration (FIG 3A). The VPAC₂ receptor selective agonist Ro 25-1553 inhibited muscle atrophy of the tibialis anterior (FIG 3A) and medial gastrocnemius (FIG 3B) induced by glucocorticoid administration. Statistical significance of the results were determined using ANCOVA.

Figure 4 (FIG. 4.) shows the results of a mouse disuse (casting) atrophy study utilizing the VPAC₂ receptor selective agonist, Ro 25-1553 (VPAC₂R agonist). Following casting of the right hind leg, male mice were injected subcutaneously in the midscapular region twice daily, with either the above indicated agent at the doses indicated in Figures 4A and 4B or vehicle control (physiological saline) for nine days. The VPAC₂R agonist was co-administered with twice daily intra-peritoneal dosing of the phosphodiesterase inhibitor theophylline (T - 30 mg/kg). On day nine, the medial gastrocnemius and tibialis anterior muscles were removed and weighed to determine the degree of atrophy. The VPAC₂ receptor selective agonist Ro 25-1553 inhibited disuse-induced atrophy of the tibialis anterior muscle (FIG 4A). In addition, Ro251553 induced

hypertrophy of the medial gastrocnemius muscle. Statistical significance of the results were determined using ANCOVA.

Figure 5 (FIG. 5.) shows the results of a rat denervation atrophy study utilizing the VPAC₂ receptor selective agonist, Ro 25-1553 (VPAC₂R receptor). Following denervation of the right sciatic nerve, male rats were injected subcutaneously in the midscapular region twice daily, with either the VPAC₂R receptor agonist at the doses indicated in Figures 5A and 5B or vehicle control (physiological saline) for nine days. The VPAC₂R receptor agonist was co-administered with twice daily intra-peritoneal dosing of the phosphodiesterase inhibitor theophylline (T - 30 mg/kg). On day nine, the tibialis anterior and extensor digitorum longus (EDL) muscles were removed and weighed to determine the degree of atrophy. This experiment demonstrated that the VPAC₂ receptor selective agonist Ro 25-1553 inhibited atrophy of the denervated tibialis anterior (FIG 5A) and EDL (FIG 5B) muscles. Statistical significance of the results were determined using ANCOVA.

15 III. Preparation of VPAC receptors, VIP or VIP Analogs, or Cell Lines Expressing VPAC Receptors

VPAC₁ receptors, VPAC₂ receptors, VIP and VIP analogs can be prepared for a variety of uses, including, but not limited to, the generation of antibodies, use as reagents in the screening assays of the present invention, and use as pharmaceutical reagents for the treatment of skeletal muscle atrophy. It will be clear to one of skill in the art, that for certain embodiments of the invention, purified polypeptides will be most useful, while for other embodiments cell lines expressing the polypeptides will be most useful. For example, in situations where it is important to retain the structural and functional characteristics of the VPAC receptor, e.g. in a screening method to identify candidate compounds which activate VPAC receptors, it is desirable to use cells which express functional VPAC receptors.

Because VIP and VIP analogs are short polypeptides, the skilled artisan will recognize that these polypeptides will be most conveniently provided by direct synthesis, rather than by recombinant means, using techniques well known in the art. In addition, many of these molecules are commercially available.

Where the source of VPAC receptors is a cell line expressing the polypeptide, the cells may, for example, endogenously express VPAC receptor, have been stimulated to increase endogenous VPAC receptor expression or have been genetically engineered to express a VPAC

receptor. Methods for determining whether a cell line expresses a polypeptide of interest are known in the art, for example, detection of the polypeptide with an appropriate antibody, use of a DNA probe to detect mRNA encoding the protein (e.g. northern blot or PCR techniques), or measuring binding of an agent selective for the polypeptide of interest (e.g. a radiolabeled selective agonist).

5 The use of recombinant DNA technology in the preparation of VPAC₁, VPAC₂, or of cell lines expressing these polypeptides is particularly contemplated. Such recombinant methods are well known in the art. To express recombinant VPAC₁ or VPAC₂ receptors, an expression vector that comprises a nucleic acid which encodes the polypeptide of interest under the control of one or more regulatory elements, is prepared. Genomic or cDNA sequences encoding VPAC₁ and VPAC₂
10 receptors from several species have been described and are readily available from the GenBank database (available at <<http://www.ncbi.nlm.nih.gov/>>). The accession numbers for VPAC₁ receptor sequences include: L13288 (human); U11079, U11080, U11081, U11082, U11083, U11084, U11085, U11086, U11087 which together make up the complete gene sequence for human VPAC₁ receptor (the assembled listing is under 806702); X75299 (human); M86835
15 (*Rattus norvegicus*); NM_011703 (*Mus musculus*), U49434 (*Sus scrofa*), AF100644 (*Rana ridibunda*), I28734 (Porcine), E05551 (*Rattus sp.*) and U56391 (*Carassius auratus*). The accession numbers for VPAC₂ receptor sequences include: X95097 (human), L40764 (human), L36566 (human), Y18423 (human), U18810 (human), D28132 (*Mus musculus*), Z25885 (*Rattus norvegicus*), U09631 (*Rattus norvegicus*), and A43808 (Rat). Using this publicly available
20 sequence information, one means of isolating a nucleic acid molecule encoding a VPAC₁ or VPAC₂ receptor is to screen a genomic DNA or cDNA library with a natural or artificially synthesized DNA probe, using methods well known in the art, e.g. by PCR amplification of the sequence from an appropriate library. Another method is to use oligonucleotide primers specific for the receptor of interest to PCR amplify the cDNA directly from mRNA isolated from a particular tissue (such
25 as skeletal muscle). Such isolated mRNA is commercially available. One of skill in the art would also recognize that by using nucleic acid probes corresponding to portions of the known VPAC receptor sequences the homologous cDNAs or genomic sequences from other species can be obtained using known methods. Particularly useful in the methods of the present invention are VPAC receptors from the species including, but not limited to, human, mouse, rat, pig, monkey,
30 chimpanzee, marmoset, dog, cow, sheep, cat, chicken and turkey. By methods well known in the art, the isolated nucleic acid molecule encoding the VPAC receptor of interest is then ligated into a suitable expression vector. The expression vector, thus prepared, is expressed in a host cell and the

host cells expressing the receptor are used directly in a screening assay or the receptor is isolated from the host cells expressing the receptor and the isolated receptor is used in a screening assay.

The host-expression vector systems that may be used for purposes of the invention include, but are not limited to: microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors containing VPAC receptor nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing VPAC receptor nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing VPAC receptor nucleotide sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, tobacco mosaic virus) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing VPAC receptor nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, HEK293, NIH3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g. retrovirus LTR) and also containing VPAC receptor nucleotide sequences.

The host cell is used to produce the polypeptide of interest. Because the VPAC receptor is a membrane bound molecule, it is purified from the host cell membranes or the VPAC receptor is utilized while anchored in the cell membrane, i.e. whole cells or membrane fractions of cells are used. Purification or enrichment of the VPAC receptors from such expression systems is accomplished using appropriate detergents and lipid micelles by methods well known to those skilled in the art.

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the gene product being expressed. For example, when a large quantity of such proteins is produced for the generation of antibodies to VPAC receptors, vectors which direct the expression of high levels of protein products, are desirable. One skilled in the art is able to generate such vector constructs and purify the proteins by a variety of methodologies including selective purification technologies such as fusion protein selective columns and antibody columns, and non-selective purification technologies.

In an insect protein expression system, the baculovirus *A. californica* nuclear polyhedrosis virus (AcNPV), is used as a vector to express foreign genes in *S. frugiperda* cells. In this case, VPAC receptor nucleotide sequences are cloned into non-essential regions of the virus and placed under the control of an AcNPV promoter. The recombinant viruses are then used to infect cells in

which the inserted gene is expressed and the protein is purified by one of many techniques known to one skilled in the art.

In mammalian host cells, a number of viral-based expression systems may be utilized. Utilization of these expression systems often requires the creation of specific initiation signals in the vectors for efficient translation of the inserted nucleotide sequences. This is particularly important if a portion of the VPAC receptor gene is used which does not contain the endogenous initiation signal. The placement of this initiation signal, in frame with the coding region of the inserted nucleotide sequence, as well as the addition of transcription and translation enhancing elements and the purification of the recombinant protein are achieved by one of many methodologies known to one skilled in the art. Also important in mammalian host cells is the selection of an appropriate cell type which is capable of the necessary post translational modifications of the recombinant protein. Such modifications, for example, cleavage, phosphorylation, glycosylation, etc., require the selection of the appropriate host cell which contains the modifying enzymes. Such host cells include, but are not limited to, CHO, HEK293, NIH3T3, COS, etc. and are known by those skilled in the art.

For long term, high expression of recombinant proteins, stable expression is preferred. For example, cell lines which stably express VPAC receptors may be engineered. One of skill in the art, following known methods such as electroporation, calcium phosphate transfection, or liposome mediated transfection, can generate a cell line which stably expresses VPAC receptors. This is usually accomplished by transfecting cells using expression vectors which contain appropriate expression control elements (e.g., promoter sequences, enhancer sequences, transcriptional termination sequences, polyadenylation sites, translational start sites, etc.), a selectable marker, and the gene of interest. The selectable marker may either be contained within the same vector, as the gene of interest, or on a separate vector which is co-transfected with the VPAC sequence containing vector. The selectable marker in the expression vector may confer resistance to the selection and allows cells to stably integrate the vector into their chromosomes and to grow to form foci which in turn can be cloned and expanded into cell lines. Alternatively the expression vector may allow selection of the cell expressing the selectable marker utilizing a physical attribute of the marker, i.e. expression of Green Fluorescent Protein (GFP) allows for selection of cells expressing the marker using fluorescence activated cell sorting (FACS) analysis.

One of skill in the art is able to select an appropriate cell type for transfection in order to allow for selection of cells into which the gene of interest has been successfully integrated. For example, where the selectable marker is herpes simplex virus thymidine kinase, hypoxanthine-

guanine phosphoribosyltransferase or adenine phosphoribosyltransferase, the appropriate cell type would be tk-, hgp⁺ or ap⁺ cells, respectively. Or, wild type cells can be used where the selectable marker is dhfr, gpt, neo or hyg⁺ which confer resistance to methotrexate, mycophenolic acid, G-418 or hygromycin, respectively. Such recombinant cell lines are useful for identification of candidate compounds that affect the VPAC receptor activity.

IV Preparation of VPAC Receptor Antibodies

Antibodies that selectively recognize one or more epitopes of a VPAC receptor are also encompassed by the invention. Such antibodies include, e.g., polyclonal antibodies, monoclonal antibodies, chimeric antibodies, human antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, molecules produced using a Fab expression library, human antibodies (polyclonal or monoclonal) produced in transgenic mice and epitope binding fragments of any of the above. For therapeutic uses, chimeric or human antibodies are preferred; human antibodies are most preferred.

The antibodies can be utilized in conjunction with the compound screening schemes described herein for the evaluation of test compounds, e.g. for immobilization of VPAC receptor polypeptides or such antibodies can be used in conjunction with gene therapy techniques to evaluate, for example, the expression of VPAC receptors either in cells or directly in patient tissues in which these genes have been introduced. In addition, antibodies of the present invention are useful in the treatment of skeletal muscle atrophy. Antibodies selective for the VPAC receptor can be screened by the methods of the present invention to identify a subset of the antibodies which are VPAC receptor agonists. In addition, anti-idiotypic antibodies generated against antibodies specific for VIP or a VIP analog may be useful as VPAC receptor agonists and like anti-VPAC antibodies may be screened for their ability to activate the VPAC receptor by methods of the present invention.

For the production of antibodies, a variety of host animals may be immunized by injection with VPAC, VIP or a VIP analog, anti-VIP antibody, anti-VIP analog antibody, or immunogenic fragments thereof by methods well known in the art. For preparation of an anti-idiotypic antibody the immunogen is an anti-VIP antibody or anti-VIP analog antibody. Production of anti-idiotypic antibodies is described, for example, in US Patent No. 4,699,880, incorporated herein by reference. Suitable host animals include, but are not limited to, rabbits, mice, goats, sheep and horses. Immunization techniques are well known in the art. Polyclonal antibodies can be purified from the serum of the immunized animals, or monoclonal antibodies can be generated by methods which are well known in the art. These techniques include, but are not limited to, the well-known hybridoma

techniques of Kohler and Milstein, human B-cell hybridoma techniques, and the EBV hybridoma technology. Monoclonal antibodies may be of any immunoglobulin class, including IgG, IgE, IgM, IgA, and IgD containing either kappa or lambda light chains.

Because of the immunogenicity of non-human antibodies in humans, chimeric antibodies
5 are preferred to non-human antibodies when used for therapeutic treatment of human patients. Techniques of producing and using chimeric antibodies are known in the art, and are described in, for example, Vaswani et al., *Ann. Allergy Asthma Immunol.*, 1998;81:105-119, Farah et al., *Critical Reviews in Eukaryotic Gene Expression*, 1998;8(3-4):321-56, U.S. Pat. Nos. 5,807,715; 4,816,397; 4,816,567; and 5,824,307, all incorporated herein by reference.

10 Completely human antibodies are particularly desirable for therapeutic treatment of human patients because they are less immunogenic than non-human antibodies. Such antibodies can be produced using transgenic mice which are substantially incapable of expressing endogenous immunoglobulin heavy and light chain genes, but which can express human heavy and light chain genes. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or
15 a portion of VPAC. Monoclonal antibodies directed against the antigen are obtained using conventional hybridoma technology from these immunized transgenic mice. This technology is described in detail in U.S. Pat. Nos. 5,874,299; 5,877,397; 5,569,825; 5,661,016; 5,770,429; and 6,075,181, all incorporated herein by reference. As an alternative to obtaining human immunoglobulins directly from the culture of the hybridoma cells, the hybridoma cells can be used
20 as a source of rearranged heavy chain and light chain loci for subsequent expression or genetic manipulation. Isolation of genes from such antibody-producing cells is straightforward since high levels of the appropriate mRNAs are available. The recovered rearranged loci can be manipulated as desired. For example, the constant region can be eliminated or exchanged for that of a different isotype or the variable regions can be linked to encode single chain Fv regions. Such techniques are
25 described in WO 96/33735 and WO 96/34096, all incorporated herein by reference.

V. Selection of Test Compounds

Compounds that can be screened in accordance with the assays of the invention include but are not limited to, libraries of known compounds, including natural products, such as plant or animal extracts, synthetic chemicals, biologically active materials including proteins, peptides such
30 as soluble peptides, including but not limited to members of random peptide libraries and combinatorial chemistry derived molecular library made of D- or L- configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed

phosphopeptide libraries), antibodies (including, but not limited to, polyclonal, monoclonal, chimeric, human, anti-idiotypic or single chain antibodies, and Fab, F(ab')₂ and Fab expression library fragments, and epitope-binding fragments thereof), organic and inorganic molecules.

In addition to the more traditional sources of test compounds, computer modeling and searching technologies permit the rational selection of test compounds by utilizing structural information from the ligand binding site of VPAC or from already identified agonists of VPAC receptors. Such rational selection of test compounds can decrease the number of test compounds that must be screened in order to identify a candidate therapeutic compound. VPAC receptors are GPCRs and thus knowledge of the VPAC protein sequence allows for the generation of a model of the binding site that can be used to screen for potential ligands. This process can be accomplished in several manners. The most robust approach involves generating a sequence alignment of the VPAC receptor sequence to a template (derived from the bacterio-rhodopsin or rhodopsin crystal structures or other GPCR model), conversion of the amino acid structures and refining the model by molecular mechanics and visual examination. If a strong sequence alignment cannot be obtained then a model may also be generated by building models of the hydrophobic helices. These are then fitted together by rotating and translating each helix relative to the others starting from the general layout of the known rhodopsin structures. Mutational data that points towards residue-residue contacts may also be used to position the helices relative to each other so that these contacts are achieved. During this process, docking of the known ligands into the binding site cavity within the helices may also be used to help position the helices by developing interactions that would stabilize the binding of the ligand. The model may be completed by refinement using molecular mechanics and loop building of the intracellular and extracellular loops using standard homology modeling techniques. General information regarding GPCR structure and modeling can be found in Schoneberg, T. et. al., *Molecular and Cellular Endocrinology*, 151 (1999), 181-193, Flower, D., *Biochimica et Biophysica Acta*, 1422 (1999), 207-234, and Sexton, P.M., *Current Opinion in Drug Discovery and Development*, 2(5), 1999, 440-448, incorporated herein by reference.

Once the model is completed, it can be used for screening for potential ligands for example by using one of several existing computer programs. The most general of these is the DOCK program (UCSF Molecular Design Institute, 533 Parnassus Ave, U-64, Box 0446, San Francisco, California 94143-0446). In several of its variants it can screen databases of commercial and/or proprietary compounds for steric fit and rough electrostatic complementarity to the binding site. It has frequently been found that molecules that score well within DOCK have a better chance of being ligands. Another program that can be used is FLEXX (Tripos Inc., 1699 South Hanley Rd.,

St. Louis, Missouri, 63144-2913 <www.tripos.com>). This program, being significantly slower, is usually restricted to searches through smaller databases of compounds. The scoring scheme within FLEXX is more detailed and usually gives a better estimate of binding ability than does DOCK. FLEXX is best used to confirm DOCK suggestions, or to examine libraries of compounds that are generated combinatorially from known ligands or templates.

VI. Screening Assays to Identify Candidate Compounds for the Regulation of Skeletal Muscle Mass or Function

The finding that VPAC receptors play a role in regulating skeletal muscle atrophy, enables various methods of screening one or more test compounds to identify candidate compounds that ultimately may be used for prophylactic or therapeutic treatment of skeletal muscle atrophy. This invention provides methods for screening test compounds for their ability to bind to VPAC receptors, activate VPAC receptors, prolong or augment the agonist-induced activation of VPAC receptors or of a VPAC receptor signal transduction pathway, increase expression of VPAC receptor genes, or increase expression of VIP or a VIP analog gene.

For screening for compounds which ultimately will be used to regulate skeletal muscle mass or function through VPAC₁ receptors in humans, it is preferred that the initial *in vitro* screen be carried out using a VPAC₁ receptor with an amino acid sequence that is greater than 60% identical to the sequence of the human VPAC₁ receptor, Accession No. L13288, (SEQ ID NO:1). More preferably the sequence of the VPAC₁ receptor is greater than 70% identical to SEQ ID NO:1, more preferably greater than 80% identical to SEQ ID NO:1 and more preferably greater than 90% identical to SEQ ID NO:1. Most preferably the test compounds will be screened against a human VPAC₁ receptor. For screening for compounds which ultimately will be used to regulate skeletal muscle mass or function through VPAC₁ in a non-human species, it is preferable to use the VPAC₁ receptor from the species in which treatment is contemplated.

For screening for compounds which ultimately will be used to regulate skeletal muscle mass or function through VPAC₂ receptors in humans, it is preferred that the initial *in vitro* screen be carried out using a VPAC₂ receptor with an amino acid sequence that is greater than 60% identical to the sequence of the human VPAC₂ receptor, Accession No. X95097, (SEQ ID NO:10). More preferably the sequence of the VPAC₂ receptor is greater than 70% identical to SEQ ID NO:10, more preferably greater than 80% identical to SEQ ID NO:10 and more preferably greater than 90% identical to SEQ ID NO:10. Most preferably the test compounds will be screened against a human VPAC₂ receptor. For screening for compounds which ultimately will be used to

regulate skeletal muscle mass or function through VPAC₂ receptors in a non-human species, it is preferable to use the VPAC₂ receptor from the species in which treatment is contemplated.

The methods of the present invention are amenable to high throughput applications, however, the use of as few as one test compound in the method is encompassed by the term screening. Test compounds which activate VPAC receptors, prolong or augment the agonist-induced activation of VPAC receptors or of a VPAC receptor signal transduction pathway, increase expression of VPAC receptor genes, or increase expression of VIP or a VIP analog gene, as determined by a method of the present invention are referred to herein as "candidate compounds". Such candidate compounds can be used to regulate skeletal muscle mass or function. However, more typically, this first level of *in vitro* screen provides a means by which to select a narrower range of compounds, i.e., the candidate compounds, which merit further investigation in additional levels of screening. The skilled artisan will recognize that a utility of the present invention is to identify, from a group of one or more test compounds, a subset of compounds which merit further investigation. One of skill in the art will also recognize that the assays of the present invention are useful in ranking the probable usefulness of a particular candidate compound relative to other candidate compounds. For instance, a candidate compound which activates VPAC at 1000 nM (but not at 10 nM) is of less interest than one which activates VPAC at 10 nM. Using such information the skilled artisan may select a subset of the candidate compounds, identified in the first level of screening, for further investigation. By the way of example only, compounds which activate VPAC at concentrations of less than 200 nM might be further tested in an animal model of skeletal muscle atrophy, whereas those above that threshold would not be further tested. The skilled artisan will also recognize that depending on how the group of test compounds is selected, and how the positives are selected, only a certain proportion of test compounds will be identified as candidate compounds, and that this proportion may be very small.

The assay systems described below may be formulated into kits comprising VPAC receptors or cells expressing the VPAC receptors which can be packaged in a variety of containers, e.g., vials, tubes microtitre well plates, bottles and the like. Other reagents can be included in separate containers and provided with the kit, e.g., positive control samples, negative control samples, buffers and cell culture media.

In one embodiment, the invention provides a method for screening one or more test compounds to identify candidate compounds that bind to VPAC receptors. Methods of determining binding of a compound to a receptor are well known in the art. Typically, the assays include the steps of incubating a source of the VPAC receptor with a labeled compound, known to bind to the

receptor, in the presence or absence of a test compound and determining the amount of bound labeled compound. The source of VPAC receptor may either be cells expressing VPAC receptors or some form of isolated VPAC receptor, as described herein. The labeled compound can be VIP or any VIP analog labeled such that it can be measured, preferably quantitatively (e.g., ¹²⁵I-labeled, europium labeled, fluorescein labeled, GFP labeled, ³⁵S-methionine labeled). Such methods of labeling are well known in the art. Test compounds that bind to the VPAC receptor cause a reduction in the amount of labeled ligand bound to the receptor, thereby reducing the signal level compared to that from control samples (absence of test compound). Variations of this technique have been described in which receptor binding in the presence and absence of G-protein uncoupling agents can discriminate agonists from antagonists (e.g. binding in the absence and presence of a guanine nucleotide analog i.e. GpppNHp). See Keen, M., *Radioligand Binding Methods for Membrane Preparations and Intact cells in Receptor Signal Transduction Protocols*, R.A.J. Challis, (ed), Humana Press Inc., Totoway N.J. (1997).

When it is desired to discriminate between compounds which bind specifically to VPAC₁ or VPAC₂ (i.e. to one receptor type but not the other) the assays described above should be conducted using a cell, or membrane from a cell, which expresses only one of the VPAC receptors, or the assays can be conducted with a recombinant source of a particular receptor. Cells expressing more than one form of VPAC may be modified using homologous recombination to inactivate or otherwise disable the VPAC receptor gene that is not of interest. Alternatively, if the source of VPAC contains more than one VPAC receptor type, the background signal produced by the receptor which is not of interest must be subtracted from the signal obtained in the assay. The background response can be determined by a number of methods, including elimination of the signal from the VPAC receptor which is not of interest by use of antisense, antibodies or selective antagonists.

In another embodiment, the invention provides methods for screening test compounds to identify candidate compounds which activate VPAC receptors. Typically, the assays are cell-based however, cell-free assays are known which are able to differentiate agonist and antagonist binding as described above. Cell-based assays include the steps of contacting cells which express the VPAC₁ or VPAC₂ receptor with a test compound or control and measuring activation of the VPAC receptor by measuring the expression or activity of components of the VPAC receptor signal transduction pathways.

As described in the background section above, VPAC receptors appear to couple through several different pathways including G_{αs}, G_{αq} or G_{αi}, depending upon the cell type. It is thought

that agonist activation of VPAC allows the receptor to signal via any of these pathways, provided that the necessary pathway components are present in the particular cell type. Thus, to screen for VPAC activation, an assay can use any of the signal transduction pathways as the readout even if the relevant cell type for treatment, *in vivo*, couples VPAC to skeletal muscle atrophy via a different pathway. One of ordinary skill in the art would recognize that a screening assay would be effective for identifying useful VPAC agonists independent of the pathway by which receptor activation was measured. Assays for measuring activation of these signaling pathways are known in the art.

For example, after contact with the test compound, lysates of the cells can be prepared and assayed for induction of cAMP. cAMP is induced in response to $G_{\alpha s}$ activation. Because $G_{\alpha s}$ is activated by receptors other than VPAC and because a test compound may be exerting its effect through VPAC receptors or by another mechanism, two control comparisons are relevant for determining whether a test compound increases levels of cAMP via activation of a VPAC receptor. One control compares the cAMP level of cells contacted with a test compound and the cAMP level of cells contacted with a control compound (i.e. the vehicle in which the test compound is dissolved). If the test compound increases cAMP levels relative to the control compound this indicates that the test compound is increasing cAMP by some mechanism. The other control compares the cAMP levels of a VPAC expressing cell line and a cell line, that is essentially the same except that it does not express the VPAC receptor where both of the cell lines have been treated with test compound. If the test compound elevates cAMP levels in the VPAC receptor expressing cell line relative to the cell line that does not express VPAC receptors, this is an indication that the test compound elevates cAMP via activation of the VPAC receptors.

In a specific embodiment of the invention, constructs containing the cAMP responsive element linked to any of a variety of reporter genes can be introduced into cells expressing VPAC receptors. Such reporter genes include, but are not limited to, chloramphenicol acetyltransferase (CAT), luciferase, glucuronide synthetase, growth hormone, fluorescent proteins (e.g., Green Fluorescent Protein), or alkaline phosphatase. Following exposure of the cells to the test compound, the level of reporter gene expression can be quantitated to determine the test compound's ability to activate the VPAC receptor.

The cells useful in this assay are the same as for the VPAC receptor binding assay described above, except that cells utilized in the activation assays preferably express a functional receptor which gives a statistically significant response to VIP or one or more VIP analog. In addition to using cells expressing full length VPAC receptors, cells can be engineered which

express VPAC receptors containing the ligand binding domain of the receptor coupled to, or physically modified to contain, reporter elements or to interact with signaling proteins. For example, a wild-type VPAC or VPAC fragment can be fused to a G-protein resulting in activation of the fused G-protein upon agonist binding to the VPAC portion of the fusion protein. Siefert, R. et al., *Trends Pharmacol. Sci.*, 1999; 20: 383-389. The cells should also preferably possess a number of characteristics, depending on the readout, to maximize the inductive response by VIP or the VIP analog, for example, for detecting a strong induction of a CRE reporter gene; (a) a low natural level of cAMP; (b) G proteins capable of interacting with VPAC receptors; (c) a high level of adenylyl cyclase; (d) a high level of protein kinase A; (e) a low level of phosphodiesterases; and (f) a high level of cAMP response element binding protein would be advantageous. To increase the response to VIP or a VIP analog, host cells could be engineered to express a greater amount of favorable factors or a lesser amount of unfavorable factors. In addition, alternative pathways for induction of the CRE reporter could be eliminated to reduce basal levels.

In some instances, G protein-coupled receptor responses subside, or become desensitized, after prolonged exposure to an agonist. Another embodiment of the invention provides methods for identifying compounds that prolong or augment the agonist-induced activation of VPAC receptors, or the VPAC receptor signal transduction pathway, in response to a VPAC receptor agonist. Such compounds may be used, for example, either alone or in conjunction with a VPAC receptor agonist, for the treatment of skeletal muscle atrophy. Typically the method uses a cell based assay comprising (i) treating cells expressing functional VPAC receptors with a VPAC receptor agonist at a concentration of agonist and for a period of agonist-receptor exposure sufficient to allow desensitization of the receptor, (ii) contacting the cells with a test compound and (iii) determining the level of activation of the VPAC receptor. One of skill in the art will recognize that several mechanisms contribute to receptor desensitization including, but not limited to, receptor phosphorylation, receptor internalization or degradation and VPAC receptor signal transduction pathway down-modulation. One of skill in the art can determine the appropriate time (i.e. before, during or after agonist treatment) for contacting the cells with the test compounds depending upon which mechanism of desensitization is targeted. For example, contacting the cells with test compounds following agonist treatment, can detect test compounds which block receptor desensitization which occurs as a result of phosphorylation of the receptor.

In another embodiment, the invention provides a method of screening one or more test compound to identify candidate compounds which regulate transcription of VPAC genes or VPAC receptor expression. Candidate compounds which regulate transcriptional activity of VPAC genes

may be identified using a reporter gene operably associated with a VPAC receptor regulatory region (reporter gene construct). Such methods are known in the art. In one such method, the reporter gene construct is contacted with a test compound in the presence of a source of cellular factors and the level of reporter gene expression is determined. A test compound which causes an increase in the level of expression, compared to a control sample, is indicative of a candidate compound which increases transcription of the VPAC gene. To provide the cellular factors required for *in vitro* or *in vivo* transcription, appropriate cells or cell extracts are prepared from any cell type that normally expresses VPAC receptors.

Candidate compounds which regulate VPAC receptor expression can also be identified in a method wherein a cell is contacted with a test compound and the expression of VPAC is determined. The level of expression of VPAC receptor in the presence of the test compound is compared with the level of expression in the absence of the test compound. Test compounds which increase the expression of VPAC are identified as candidate compounds for increasing muscle mass or muscle function. Such a method detects candidate compounds which increase the transcription or translation of the VPAC receptor or which increase the stability of the mRNA or VPAC receptors.

In another embodiment, this invention provides methods for screening one or more test compounds to identify candidate compounds which regulate the expression of the VIP or a VIP analog. Such assays are performed essentially as described above for the assays to identify candidate compounds which regulate expression of VPAC receptors with the following modifications. To identify candidate compound which regulate transcription from the VIP gene or a VIP analog gene, the reporter gene is operably associated with the regulatory region of the VIP gene or VIP analog gene of interest and the source of cellular factors should be from a cell type that expresses the gene of interest.

VII. Screening of candidate compounds using models of skeletal muscle atrophy

Candidate compounds selected from one or more test compounds by an *in vitro* assay, as described above, can be further tested for their ability to regulate skeletal muscle mass or function in model systems of skeletal muscle atrophy and/or hypertrophy. Such models of skeletal muscle atrophy or hypertrophy include both *in vitro* cell culture models and *in vivo* animal models of skeletal muscle atrophy. Such additional levels of screening are useful to further narrow the range of candidate compounds that merit additional investigation, e.g. clinical trials.

Cell Culture Models of Muscle Atrophy

In vitro models of skeletal muscle atrophy are known in the art. Such models are described, for example, in ; Vandenburg, H.H., *In Vitro* 24: 609-619, 1988; Vandenburg, H.H. et al., *J of Biomechanics*, 24 Suppl 1: 91-9, 1991; Vandenburg, H.H et al., *In Vitro Cell. Dev. Biol.*, 24(3): 166-74, 1988; Chromiak, J.A., et al., *In Vitro Cell. Dev. Biol. Anim.*, 34(9): 694-703, 1998; Shansky, J., et al., *In Vitro Cell. Dev. Biol. Anim.*, 33(9): 659-61, 1997; Perrone, C.E. et al., *J. Biol. Chem.* 270(5): 2099-106, 1995; Chromiac, J.A. and Vandenburg, H.H., *J. Cell. Physiol.* 159(3): 407-14, 1994; and Vandenburg, H.H. and Karlisch, P., *In Vitro Cell. Dev. Biol.*, 25(7): 607-16, 1989. Such models are useful, but not required, following the *in vitro* screening described above in order to further narrow the range of candidate compounds that merit testing in an animal model. Cell culture models are treated with candidate compounds and the response of the model to the treatment is measured by assessing changes in muscle markers such as: muscle protein synthesis or degradation, changes in skeletal muscle mass or contractile function. Those compounds which induce significant changes in the muscle markers are typically screened further in an animal model of skeletal muscle atrophy.

Animal Models of Skeletal Muscle Atrophy

The candidate compounds are administered to non-human animals and the response of the animals is monitored, for example, by assessing changes in markers of atrophy or hypertrophy such as: skeletal muscle mass, skeletal muscle function, muscle or myofiber cross-sectional area, contractile protein content, non-contractile protein content or a biochemical or genetic marker that correlates with skeletal muscle mass or function changes. Candidate compounds which induce skeletal muscle hypertrophy or prevent any aspect of skeletal muscle atrophy should be considered as prospective therapeutic candidates for treatment of human skeletal muscle atrophy, and are referred to herein as candidate therapeutic compounds. In addition to assessing the ability of a candidate compound to regulate skeletal muscle atrophy, undesirable side effects such as toxicity may also be detected in such a screen. The absence of unacceptably high levels of side effects may be used as a further criterion for the selection of candidate therapeutic compounds.

A variety of animal models for skeletal muscle atrophy are known in the art, such as those described in the following references: Herbison, G.J., et al. (1979) *Arch. Phys. Med. Rehabil.* 60:401-404, Appell, H-J. (1990) *Sports Medicine* 10:42-58, Hasselgren, P-O. and Fischer, J.E. (1998) *World J. Surg.* 22:203-208, Agbenyega, E.T. and Wareham, A.C. (1992) *Comp. Biochem. Physiol.* 102A:141-145, Thomason, D.B. and Booth, F.W. (1990) *J. Appl. Physiol.* 68:1-12, Fitts,

R.H., et al. (1986) *J. Appl. Physiol.* 60:1946-1953, Bramanti, P., et al. (1998) *Int. J. Anat. Embryol.* 103:45-64, Cartee, G.D. (1995) *J. Gerontol. A Biol. Sci. Med. Sci.* 50:137-141, Cork, L.C., et al. (1987) *Prog. Clin. Biol. Res.* 229:241-269, Booth, F.W. and Gollnick, P.D. (1983) *Med. Sci. Sports Exerc.* 15:415-420, Bloomfield, S.A. (1997) *Med. Sci. Sports Exerc.* 29:197-206.

- 5 Preferred animals for these models are mice and rats. These models include, for example, models of disuse-induced atrophy such as casting or otherwise immobilizing limbs, hind limb suspension, complete animal immobilization, and reduced gravity situations. Models of nerve damage induced atrophy include, for example, nerve crush, removal of sections of nerves which innervate specific muscles, toxin application to nerves and infection of nerves with viral, bacterial or eukaryotic
- 10 infectious agents. Models of glucocorticoid-induced atrophy include application of atrophy-inducing doses of exogenous glucocorticoid to animals, and stimulation of endogenous corticosteroid production, for example, by application of hormones which activate the hypothalamus-pituitary-adrenal (HPA) axis. Models of sepsis-induced atrophy include, for example, inoculation with sepsis-inducing organisms such as bacteria, treatment of the animal with
- 15 immune-activating compounds such as bacterial cell wall extract or endotoxin, and puncture of intestinal walls. Models of cachexia-induced atrophy include, for example, inoculation of an animal with tumorigenic cells with cachexia forming potential, infection of an animal with infectious agents (such as viruses which cause AIDS) which result in cachexia and treatment of an animal with hormones or cytokines such as CNTF, TNF, IL-6, IL-1, etc. which induce cachexia.
- 20 Models of heart failure-induced atrophy include the manipulation of an animal so that heart failure occurs with concomitant skeletal muscle atrophy. Neurodegenerative disease-induced atrophy models include autoimmune animal models such as those resulting from immunization of an animal with neuronal components. Muscular dystrophy-induced models of atrophy include natural or man-made genetically-induced models of muscular dystrophy such as the mutation of the
- 25 dystrophin gene which occurs in the Mdx mouse.

Animal models of skeletal muscle hypertrophy include, for example, models of increased limb muscle use due to inactivation of the opposing limb, reweighting following a disuse atrophy inducing event, reutilization of a muscle which atrophied because of transient nerve damage, increased use of selective muscles due to inactivation of a synergistic muscle (e.g. compensatory

- 30 hypertrophy), increased muscle utilization due to increased load placed on the muscle and hypertrophy resulting from removal of the glucocorticoid after glucocorticoid-induced atrophy. Preferred animal atrophy models include the sciatic nerve denervation atrophy model,

glucocorticoid-induced atrophy model, and the leg casting disuse atrophy model which are described in further detail below.

5 The sciatic nerve denervation atrophy model involves anesthetizing the animal followed by the surgical removal of a short segment of either the right or left sciatic nerve, e.g. in mice the sciatic nerve is isolated approximately at the midpoint along the femur and a 3-5 mm segment is removed. This denervates the lower hind limb musculature resulting in atrophy of these muscles. Typically, innervation to the biceps femoris is left intact to provide satisfactory motion of the knee for virtually normal ambulation. Typically, in untreated animals, muscle mass of the denervated muscles is reduced 30-50% ten days following denervation. Following denervation, test
10 compounds are administered e.g., by injection or by continuous infusion, e.g. via implantation of an osmotic minipump (e.g. Alza, Palo Alto, CA), to determine their effect on denervation induced skeletal muscle atrophy. At various times following denervation, the animals are euthanized and lower leg muscles are dissected rapidly from both the denervated and nondenervated legs, the muscles, cleaned of tendons and connective tissue, are weighed. The extent of atrophy in the
15 affected muscles is analyzed, for example, by measuring muscle mass, muscle cross-sectional area, myofiber cross-sectional area or contractile protein content.

The glucocorticoid induced atrophy model involves the administration of a glucocorticoid to the test animal, e.g. 1.2 mg/kg/day of dexamethasone in the drinking water. Typically, in
20 untreated animals, skeletal muscle mass is reduced 30-50% following ten days of dexamethasone administration. Concomitantly with, or following glucocorticoid administration, test compounds are administered e.g., by injection or by continuous infusion to determine their effect on glucocorticoid induced skeletal muscle atrophy. At various times following glucocorticoid administration, the extent of atrophy in the affected muscles is analyzed as described above for the denervation model.

25 The leg casting disuse atrophy model involves casting one hind leg of an animal from the knee down through the foot. Typically, muscle mass is reduced 20-40% after ten days of casting. Following casting, test compounds are administered by injection or by continuous infusion via implantation of an osmotic minipump (e.g. Alza, Palo Alto, CA) to determine their effect on leg casting induced skeletal muscle atrophy. At various times following leg casting, the extent of
30 atrophy in the affected muscles is analyzed as described above for the denervation model.

One of skill in the art would recognize that in screening for compounds for human use, because there are differences between the human VPAC receptor and the VPAC receptor from other animal species, there may be some false positive or negative results which arise when the

screen is carried out using non-human VPAC receptor. Thus, it is preferable to do the initial *in vitro* screen using human VPAC receptors. In certain circumstances, identified candidate compounds may be active toward only the human receptor and not toward a non-human receptor. In such circumstances, it may still be desirable to determine whether these candidate compounds are able to regulate skeletal muscle mass or function in a second level of screening. Because these candidates do not activate non-human VPAC, a standard *in vivo* screen with non-human animal is not advised. In such circumstances the second level of screening for these candidates may be performed in transgenic animals that express human VPAC receptors.

Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, goats, dogs and non-human primates may be used to generate VPAC receptor transgenic animals. Mice and rats are preferred, mice are most preferred. A variety of techniques are known in the art and may be used to introduce the human VPAC transgenes into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection, retrovirus-mediated gene transfer into germ lines, gene targeting in embryonic stem cells, electroporation of embryos and sperm-mediated gene transfer.

VIII. Gene Therapy Methods for the Treatment of Skeletal Muscle Atrophy

The expression of VPAC, VIP and VIP analogs can be controlled *in vivo* using gene therapy approaches. VPAC, VIP and VIP analog gene expression and/or activity can be increased by introducing a VPAC receptor, a constitutively active VPAC, VIP or VIP analog gene, into the appropriate tissue. Overexpression of these genes will increase the total cellular VPAC activity, thus, regulating skeletal muscle atrophy. The gene or genes of interest are inserted into a vector suitable for expression in the subject. These vectors include, but are not limited to, adenovirus, adenovirus associated virus, retrovirus and herpes virus vectors in addition to other particles that introduced DNA into cells (e.g. liposome, gold particles, etc.) or by direct injection of the DNA expression vector, containing the gene of interest, into human tissue (e.g. muscle).

Additional methods which may be utilized to increase the overall level of VIP or VIP analog gene expression include the introduction of VIP or VIP analog-expressing cells, preferably autologous cells, into the patient. These cells may be either recombinant (e.g. engineered to express VIP or VIP analogs) or non-recombinant (e.g. cells which endogenously express VIP or VIP analogs). The cells can be delivered to multiple sites in the body (e.g. muscle) for treatment of skeletal muscle atrophy. The technologies of cell-based gene therapy are known to those skilled in the art.

IX. Pharmaceutical Formulations and Methods for Use

Candidate compounds or candidate therapeutic compounds identified by screening methods described herein, can be administered to individuals to treat skeletal muscle atrophy. To this end, the present invention encompasses methods and compositions for modulating skeletal muscle atrophy, including, but not limited to, skeletal muscle atrophy induced by disuse due to surgery, bed rest, broken bones; denervation/nerve damage due to spinal cord injury; autoimmune disease; infectious disease; glucocorticoid use for unrelated conditions; sepsis due to infection or other causes; nutrient limitation due to illness or starvation; cancer cachexia; chronic inflammation; AIDS cachexia; COPD; congestive heart failure; sarcopenia and genetic disorders; e.g. muscular dystrophies, neurodegenerative diseases. Agonists of VPAC can be used to inhibit skeletal muscle atrophy. It is not necessary that effective compounds demonstrate absolute specificity for VPAC or for the VPAC receptor subtype of interest. It is contemplated that specific antagonist of other affected receptors can be co-administered with an effective, but nonspecific, agonist. Alternately, this lack of specificity may be addressed by modulation of dose alone, or the dosing regimen.

The candidate compounds or candidate therapeutic compounds identified by the screening methods of the present invention may be administered in conjunction with compounds which prolong or augment the activation of a VPAC receptor or of a VPAC receptor signal transduction pathway. These may be known compounds, for example, theophylline, or these compounds may be identified by the screening methods of this invention to prolong or augment the activation of a VPAC receptor or of a VPAC receptor signal transduction pathway.

Dose Determinations

Safety and therapeutic efficacy of compounds which agonize VPAC can be determined by standard procedures using either *in vitro* or *in vivo* technologies. Compounds which exhibit large therapeutic indices are preferred, although compounds with lower therapeutic indices are useful if the level of side effects is acceptable. The data obtained from the *in vitro* and *in vivo* toxicological and pharmacological techniques can be used to formulate the human range of doses which may be useful. The preferred dose lies in the range in which the circulating concentration of the compound is therapeutically maximal with acceptable safety. The circulating concentration of the compound may vary depending on the dose form, time after dosing, route of administration, etc. Doses outside this range are also useful provided the side effects are acceptable. Such matters as age and weight of the patient, and the like, can be used to determine such matters in the conventional

manner. Pharmacogenetic approaches may be useful in optimizing compound selection, doses and dosing regimen in clinical populations.

Formulation and Use

Pharmaceutical compositions for use in the modulation of skeletal muscle atrophy in accordance with the present invention may be formulated using conventional methodologies using pharmaceutically acceptable carriers and excipients. The compositions of this invention are preferably provided in unit dosage form. As used herein, a "unit dosage form" is a composition of this invention containing an amount of a VPAC receptor agonist that is suitable for administration to an animal, preferably a mammal, more preferably a human subject, in a single dose, according to good medical practice. Pharmaceutical compositions may be formulated for delivery by, for example, intranasal, transdermal, inhalation, parenteral, cutaneous, oral or rectal administration. For oral administration, the pharmaceutical composition may take the form of tablets or capsules containing the pharmacologically active compound and additives including, but not limited to, binding agents, fillers, lubricants, disintegrants, or wetting agents. The tablets may be coated. Liquid preparations for oral administration include, but are not limited to, syrups, suspensions or dry products which are reconstituted with liquid vehicle before use, containing the pharmacologically active compound and additives including, but not limited to, suspending agents, emulsifying agents, non-aqueous vehicles, preservatives, buffer salts, flavoring, coloring, sweetening agents, etc. Pharmaceutical compositions for oral administration may be formulated for controlled release of the pharmacologically active compounds either in the mouth, stomach or intestinal tract.

For inhalation administration, the compounds for use according to the present invention may be delivered by, but not limited to, the following forms: liquid, powder, gel or in the form of an aerosol spray utilizing either pressurized or non-pressurized propellants in either premeasured or non-premeasured doses. The pharmacologically active compound may be formulated with appropriate fillers, vehicles, preservatives, buffers, etc. For parenteral administration, the pharmacologically active compound may be formulated with acceptable physiological carriers, preservatives, etc. and be prepared as suspensions, solutions, emulsion, powders ready for constitution, etc. for either bolus injection or infusion. Doses of these compounds may be administered by a variety of technologies including hypodermic needles, high pressure devices, etc. For rectal administration, the pharmacologically active compound may be formulated with acceptable physiological carriers, preservatives, etc. for delivery as suppositories, enemas, etc. For

cutaneous administration, the pharmacologically active compound may be formulated with acceptable physiological carriers including lotions, emollients, etc. or incorporated into a patch type device. For long term administration, the pharmacologically active compound and appropriate additives such as, but limited to, polymers, hydrophobic materials, resins, etc. may be formulated as a depot preparation for either injection or implantation at multiple sites including but not limited to intramuscular and subcutaneous locations. In addition, the pharmacologically active compound may be administered by a dispensing device.

Monitoring of Effects During Clinical Trials

Monitoring the influence of compounds (e.g. drugs) on the expression or activity of VPAC can be employed not only in basic drug screening, but also in clinical trials. For example, the effectiveness of a compound determined by a screening assay to increase VPAC receptor activity or VPAC receptor expression can be assessed in clinical trials of patients with, or at risk for, skeletal muscle atrophy. At various times following administration of the test compound or placebo, the effect of the compound on the patient can be determined, for example, by observing the change in skeletal muscle mass, skeletal muscle function, biochemical markers of muscle breakdown or quality of life measures. Methods of measuring skeletal muscle mass in human subjects are known in the art and include, for example: measuring the girth of a limb; measuring muscle thickness with for instance, computer tomography, MRI or supersonics; or muscle biopsy to examine morphological and biochemical parameters (e.g. cross-section fiber area, fiber diameter or enzyme activities). Furthermore, because skeletal muscle mass is correlated with skeletal muscle function, muscle function can be used as a surrogate marker of mass and muscle mass changes can be assessed using functional measurements, e.g. strength, the force of a group of synergist muscles, or contraction characteristics found in electromyographic recordings. In addition, muscle protein loss as a result of muscle atrophy can be measured by quantitating levels of amino acids or amino acids derivatives, i.e. 3-methyl histidine, in the urine or blood of a subject. For a review of such methods see Appell, *Sports Med.* 10:42-58 (1990). Quality of life measures include, but are not limited to, the ease of getting out of a chair, number of steps taken before tiring or ability to climb stairs.

EXAMPLES

Example 1. Construction of vectors for human VPAC₂ receptor expression.

The human VPAC₂ receptor (hVPAC₂R) DNA sequence (Accession No. X95097) is retrieved and two oligonucleotides including one containing the 5' end of the gene beginning at the

initiation codon (5' oligonucleotide) and one containing the 3' end of the gene containing the stop codon (3' oligonucleotide) are synthesized. These oligonucleotides are designed to contain restriction endonuclease sites which are not present in the hVPAC₂ gene with one unique site in the 5' oligonucleotide and a different unique restriction endonuclease site in the 3' oligonucleotide. In addition, the 3' oligonucleotide contains a polyadenylation addition signal sequence. Double stranded cDNA from human skeletal muscle is purchased from the Universal QUICK-Clone cDNA collection (Clontech Inc., Palo Alto, CA, USA). Using the above 5' and 3' oligonucleotides, the hVPAC₂R cDNA is amplified by PCR of the human skeletal muscle cDNA using the AdvanTaq PCR kit (Clontech Inc., Palo Alto, CA, USA). The hVPAC₂ gene PCR product is purified from PCR artifacts by agarose gel electrophoresis and the hVPAC₂ gene DNA fragment is purified from the agarose gel using a purification product such as NucleoTrap (Clontech Inc., Palo Alto, CA, USA).

Cloning of the hVPAC₂R PCR product into the pIRESneo vector (Clontech Inc., Palo Alto, CA, USA) is accomplished by first cutting the hVPAC₂R PCR product and the pIRESneo vector with the appropriate restriction endonucleases so that the 5' and 3' restriction endonuclease sites are ready for ligation. The pIRESneo vector DNA is ligated to the hVPAC₂R PCR product DNA using DNA ligase, from the AdvantAgeTMPCR Cloning Kit (Clontech Inc., Palo Alto, CA, USA), according to the manufacturer's recommendations. The ligated vector and insert construct (pIRESneo/ hVPAC₂R) is then used to transform TOP10F' competent E. coli cells (Clontech Inc., Palo Alto, CA, USA). Transformed cells are plated on LB/X-gal/IPTG plus ampicillin containing agar. White colonies (positive clones) are selected and individually cultured in LB medium. Plasmid DNA is isolated using NucleoBond DNA Purification System (Clontech Inc., Palo Alto, CA, USA). The insert from at least one clone is sequenced to ensure that the hVPAC₂R sequence is correct. HEK293 cells containing a stably integrated Mercury CRE-LUC plasmid (Clontech Inc., Palo Alto, CA, USA) are transfected with purified pIRESneo/ hVPAC₂R DNA, having the correct sequence insert, utilizing the CalPhosTM Mammalian Transfection Kit (Clontech Inc., Palo Alto, CA, USA). Cells stably transfected with pIRESneo/ hVPAC₂R DNA are selected by culturing the cells in G418. The stably transfected cells (HEK293/CRE-LUC/pIRESneo/ hVPAC₂R cells) are propagated in DMEM (Life Technologies, Rockville, MD) containing 10% fetal bovine serum (Clontech Inc., Palo Alto, CA, USA), penicillin/streptomycin solution (Life Technologies, Rockville, MD), L-glutamine (Life Technologies, Rockville, MD), and non-essential amino acid (Life Technologies, Rockville, MD) at 37°C in a 5% carbon dioxide/95% air atmosphere. The clones are characterized for both VIP binding and CRE-LUC activation following

exposure to VIP as described in Example 2 and Example 3. Cells expressing the hVPAC₂ receptor at an appropriate level and which are appropriately coupled to the CRE-LUC reporter system are then utilized for further analysis.

5 Example 2. Receptor Binding Assays

Receptor binding analysis of compounds is performed in whole cells by plating the HEK293/CRE-LUC/pIRESneo/ hVPAC₂R cells from Example 1 in a 96 well polylysine coated plate. Cells are seeded in DMEM medium containing 10% fetal bovine serum, penicillin/streptomycin solution, L-glutamine, and non-essential amino acid at 37°C in a 5% carbon dioxide/95% air atmosphere and
10 incubated overnight. The culture medium is removed and the appropriate amount of VIP covalently labeled with Europium (Eu-VIP) in MEM (Life Technologies, Rockville, MD) + 10% Seablock (Clonetech Inc., Palo Alto, CA, USA) is added. The cells are incubated with the Eu-VIP for 90 minutes at room temperature then washed 4 times with phosphate buffered saline lacking magnesium and calcium (Life Technologies, Rockville, MD). Following the final wash,
15 enhancement solution is added (Wallac Inc., Gaithersburg, MD) and the plate is read on a Wallac plate reader (Wallac Inc., Gaithersburg, MD) using the BioWorks Europium program. For saturation binding analysis, log doses of Eu-VIP ranging from 10⁻¹² to 10⁻³ M are added to the cells and binding analyzed both in the absence and the presence of a saturating concentration of unlabeled VIP for evaluation of non-specific binding. For competitive binding, a concentration of
20 Eu-VIP is added which is half maximal, in terms of binding, in addition to varying concentrations of the compound of interest.

Example 3. Receptor Activation Assay

Receptor activation analysis is performed by seeding the HEK293/CRE-LUC/pIRESneo/
25 hVPAC₂R cells of Example 1 into Packard View Plate-96 (Packard Inc., CA). Cells are seeded in DMEM medium containing 10% fetal bovine serum, penicillin/streptomycin solution, L-glutamine, and non-essential amino acid at 37°C in a 5% carbon dioxide/95% air atmosphere and incubated overnight. The medium is then removed and replaced with DMEM (Life Technologies, Rockville, MD) containing 0.01% bovine albumin fraction V (SIGMA, St. Louis, MO) containing the
30 compound of interest. The cells are then incubated for four hours at 37°C in a 5% carbon dioxide/95% air atmosphere after which the medium is removed and the cells are washed twice with Hanks Balanced Salt Solution (Life Technologies, Rockville, MD). Lysis Reagent (Promega Inc., Madison, WI) is then added to the washed cells and the cells are incubated for 20 minutes at 37°C

in a 5% carbon dioxide/95% air atmosphere. The cells are then placed at -80°C for 20 minutes followed by a 20 minute incubation at 37°C in a 5% carbon dioxide/95% air atmosphere. After this incubation, Luciferase Assay Buffer and Luciferase Assay Substrate (Promega Inc., Madison, WI) are added to the cell lysates and luciferase activity quantitated using a luminometer. Relative activity of a compound is evaluated by comparing the increase following exposure to compound to the level of luciferase in HEK cells which contain the CRE-LUC construct without the hVPAC₂ receptor following exposure to compound. Specificity of response is also checked by evaluating luciferase response of hVPAC₂ receptor/CRE-LUC HEK cells to compound in the presence and absence of a 10-fold excess of hVPAC₂ receptor antagonist.

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Example 4. Screen to identify candidate compounds that prolong or augment the activation of hVPAC₂ receptors or of a VPAC₂ receptor signal transduction pathway.

Identification of compounds that prolong or augment the agonist-induced activation of VPAC₂ receptors, or of a VPAC₂ receptor signal transduction pathway, involves a variation of the Receptor Activation Assay described in Example 3. Specifically, this assay is performed by seeding the HEK293/ CRE-LUC/pIRESneo/hVPAC₂R receptor cells into Packard View Plate-96 (Packard Inc., CA). Cells are seeded in DMEM medium containing 10% fetal bovine serum, penicillin/streptomycin solution, L-glutamine, non-essential amino acid, and saturating amounts of VIP at 37°C in a 5% carbon dioxide/95% air atmosphere and incubated for 48 hours. The medium is then removed and replaced with DMEM (Life Technologies, Rockville, MD) containing 0.01% bovine albumin fraction V (SIGMA, St. Louis, MO) and VIP in addition to the compound of interest. The cells are then incubated for four hours at 37°C in a 5% carbon dioxide/95% air atmosphere after which the medium is removed and the cells are washed twice with Hanks Balanced Salt Solution (Life Technologies, Rockville, MD). Lysis Reagent (Promega Inc., Madison, WI) is then added to the washed cells and the cells are incubated for 20 minutes at 37°C in a 5% carbon dioxide/95% air atmosphere. The cells are then placed at -80°C for 20 minutes followed by a 20 minute incubation at 37°C in a 5% carbon dioxide/95% air atmosphere. After this incubation, Luciferase Assay Buffer and Luciferase Assay Substrate (Promega Inc., Madison, WI) are added to the cell lysates and luciferase activity is quantitated using a luminometer. Test compounds which stimulate fluorescence significantly above the levels of control untreated cells, after correction for variations in cell density, are considered candidate compounds for regulating skeletal muscle mass or function. The compounds of most interest are those which induce relatively higher levels of fluorescence.

Example 5. Screens to identify candidate compounds that increase hVPAC₂ receptor expression

The sequence containing the promoter region of the hVPAC₂ receptor gene, beginning far enough upstream of the transcriptional initiation site to contain all the regulatory elements necessary for physiological expression of the hVPAC₂ receptor gene in the appropriate tissue is retrieved from the human genome database. Two oligonucleotides, one containing the 5' end of the promoter region (5' oligonucleotide) and one containing the 3' end of the promoter region including the transcriptional start site (3' oligonucleotide) are synthesized. These oligonucleotides also contain restriction endonuclease sites which are not present in the hVPAC₂ gene regulatory region with one unique site in the 5' oligonucleotide and a different unique restriction endonuclease site in the 3' oligonucleotide. The 5' and 3' oligonucleotides are used for PCR amplification of the hVPAC₂ gene regulatory region from human DNA (Clontech Inc., Palo Alto, CA, USA) using the PCR kit, Advantage® Genomic PCR kit (Clontech Inc., Palo Alto, CA, USA). The hVPAC₂ gene regulatory region PCR product is purified from PCR artifacts by agarose gel electrophoresis and the hVPAC₂ gene regulatory region DNA fragment is purified from the agarose gel using a purification product such as NucleoTrap (Clontech Inc., Palo Alto, CA, USA). Cloning of the hVPAC₂ gene regulatory region PCR product into the pECFP-1 vector (Clontech Inc., Palo Alto, CA, USA) is accomplished by first cutting the hVPAC₂ gene regulatory region PCR product and the pECFP-1 vector with the appropriate restriction endonucleases so that the 5' and 3' restriction endonuclease sites are ready for ligation. Ligation of the pECFP-1 vector DNA to the hVPAC₂ gene regulatory region PCR product DNA is accomplished using DNA ligase from the AdvantAge™ PCR Cloning Kit (Clontech Inc., Palo Alto, CA, USA) according to the manufacturer's recommendations. The ligated vector and insert construct is then used to transform TOP10F' competent E. coli cells (Clontech Inc., Palo Alto, CA, USA). The cells are plated on LB plus kanamycin containing agar and kanamycin resistant colonies are selected for further analysis. Kanamycin resistant clones are cultured in LB containing kanamycin medium and plasmid DNA is isolated using NucleoBond DNA Purification System (Clontech Inc., Palo Alto, CA, USA) and the construct containing the hVPAC₂ gene regulatory region is analyzed by DNA sequencing to ensure construct correctness and integrity. Purified construct plasmid DNA containing the hVPAC₂ gene regulatory region is then transfected into the HEK293 cells utilizing calcium phosphate-mediated transfection utilizing the CalPhos™ Mammalian Transfection Kit (Clontech Inc., Palo Alto, CA, USA). Transfected cell clones are selected using G418, isolated

and propagated in DMEM (Life Technologies, Rockville, MD) containing 10% fetal bovine serum (Clonotech Inc., Palo Alto, CA, USA), penicillin/streptomycin solution (Life Technologies, Rockville, MD), L-glutamine (Life Technologies, Rockville, MD), non-essential amino acid (Life Technologies, Rockville, MD) and G418 (Life Technologies, Rockville, MD) at 37°C in a 5% carbon dioxide/95% air atmosphere. G418 resistant clones are characterized by Southern blotting to ensure that they contain the hVPAC₂ gene promoter sequence; in addition activation of the hVPAC₂ gene regulatory region is analyzed using an appropriate stimulating agent. Cells expressing the hVPAC₂ gene regulatory region-ECFP at an appropriate level are then used in assays designed to evaluate compounds which can modulate the activity of the hVPAC₂ gene regulatory region as follows. The regulatory region activation analysis is performed by seeding the hVPAC₂ gene regulatory region-ECFP containing HEK293 cells at an appropriate density into black with clear bottom 96 well microtiter plates and allowed to grow overnight. The following day, the medium is removed and the test compound added in fresh growth medium. The cells are incubated for 16 hours at 37°C in a 5% carbon dioxide/95% air atmosphere followed by measurement of fluorescence (excitation at 433 (453) nm by detecting emission at 475(501) nm using a fluorometer (biolumin™ 960, Molecular Dynamics/Amersham Pharmacia Biotech, Piscataway, NJ). Test compounds which stimulate fluorescence significantly above the levels of control untreated cells, after correction for variations in cell density, are considered candidate compounds for regulating skeletal muscle mass or function. The compounds of most interest are those which induce relatively higher levels of fluorescence.

Example 6. Screens to identify compounds that increase human VIP expression

The methods for identifying compounds that increase human VIP (hVIP) expression are essentially identical to those for identifying compounds which increase hVPAC₂ receptor expression except the regulatory region used is that for the hVIP gene. The sequence containing the regulatory region of the hVIP gene, beginning far enough upstream of the transcriptional initiation site to contain all the regulatory elements necessary for physiological expression of the hVIP gene in the appropriate tissue is retrieved from the human genome database. Two oligonucleotides, one containing the 5' end of the regulatory region (5' oligonucleotide) and one containing the 3' end of the regulatory region including the transcriptional start site (3' oligonucleotide) are synthesized. These oligonucleotides also contain restriction endonuclease sites which are not present in the hVIP gene regulatory region with one unique site in the 5' oligonucleotide and a different unique restriction endonuclease site in the 3' oligonucleotide. The 5' and 3' oligonucleotides are used for

PCR amplification of the hVIP gene regulatory region from human DNA (Clonotech Inc., Palo Alto, CA, USA) using the Advantage® Genomic PCR kit (Clonotech Inc., Palo Alto, CA, USA). The hVIP gene regulatory region PCR product is purified from PCR artifacts by agarose gel electrophoresis and the hVIP gene regulatory region DNA fragment is purified from the agarose gel using the purification product, NucleoTrap (Clonotech Inc., Palo Alto, CA, USA). Cloning of the hVIP gene regulatory region PCR product into the pECFP-1 vector (Clonotech Inc., Palo Alto, CA, USA) is accomplished by first cutting the hVIP gene regulatory region PCR product and the pECFP-1 vector with the appropriate restriction endonucleases so that the 5' and 3' restriction endonuclease sites are ready for ligation. Ligation of the pECFP-1 vector DNA to the hVIP gene regulatory region PCR product DNA is accomplished using DNA ligase from AdvantAge™ PCR Cloning Kit (Clonotech Inc., Palo Alto, CA, USA) according to the manufacturer's recommendations. The ligated vector and insert construct is then used to transform TOP10F' competent E. coli cells (Clonotech Inc., Palo Alto, CA, USA). The cells are plated on LB plus kanamycin containing agar and kanamycin resistant colonies are selected for further analysis.

Kanamycin resistant clones are cultured in LB containing kanamycin medium and plasmid DNA is isolated using NucleoBond DNA Purification System (Clonotech Inc., Palo Alto, CA, USA) and the construct containing the hVIP gene regulatory region is analyzed by DNA sequencing to ensure construct correctness and integrity. Purified construct plasmid DNA containing the hVIP gene regulatory region is then transfected into the HEK293 cells utilizing calcium phosphate-mediated transfection utilizing the CalPhos™ Mammalian Transfection Kit (Clonotech Inc., Palo Alto, CA, USA). Transfected cell clones are selected using G418, isolated and propagated in DMEM (Life Technologies, Rockville, MD) containing 10% fetal bovine serum (Clonotech Inc., Palo Alto, CA, USA), penicillin/streptomycin solution (Life Technologies, Rockville, MD), L-glutamine (Life Technologies, Rockville, MD), non-essential amino acid (Life Technologies, Rockville, MD) and G418 (Life Technologies, Rockville, MD) at 37°C in a 5% carbon dioxide/95% air atmosphere. G418 resistant clones are characterized by Southern blotting to ensure that they contain the hVIP gene regulatory region sequence; in addition activation of the hVIP gene regulatory region is analyzed using an appropriate stimulating agent. Cells expressing the hVIP gene regulatory region-ECFP at an appropriate level are then used in assays designed to evaluate compounds which can modulate the activity of the hVIP gene regulatory region as follows. The regulatory region activation analysis is performed as in Example 5 except that clones containing the hVIP gene regulatory region construct are used.

Example 7. Method of making human antibodies which activate the hVPAC₂ receptor.

Fully human monoclonal antibodies which activate the hVPAC₂ receptor are produced by first generating recombinant hVPAC₂ receptor protein as follows. The procedure from Example 1 is followed to obtain the hVPAC₂ PCR product. This hVPAC₂ PCR product is then cloned into the

5 pHAT20 vector (Clontech Inc., Palo Alto, CA, USA) by first cutting the hVPAC₂ gene PCR product and the pHAT20 vector with the appropriate restriction endonucleases so that the 5' and 3' restriction endonuclease sites are ready for ligation. Ligation of the pHAT20 vector DNA to the hVPAC₂ gene PCR product DNA is accomplished using DNA ligase from the AdvantAge™PCR Cloning Kit (Clontech Inc., Palo Alto, CA, USA) according to the manufacturer's

10 recommendations. The ligated vector/insert construct is then used to transform TOP10F' competent E. coli cells (Clontech Inc., Palo Alto, CA, USA). Transformed cells are plated on LB plus ampicillin containing agar and ampicillin resistant colonies are selected for further analysis. Positive clones are cultured in LB medium containing ampicillin and plasmid DNA is isolated using NucleoBond DNA Purification System (Clontech Inc., Palo Alto, CA, USA) and the construct

15 containing the hVPAC₂ gene is analyzed by DNA sequencing to ensure construct correctness and integrity. The hVPAC₂-pHAT20 vector DNA is then used for additional PCR cloning by utilizing a 5' oligonucleotide containing the beginning of the HAT sequence and a unique restriction endonuclease site not present in the hVPAC₂-pHAT20 construct and the 3' hVPAC₂ oligonucleotide utilized previously. The oligonucleotide primers are used to PCR amplify the

20 HAT-hVPAC₂ fusion gene from the hVPAC₂-pHAT20 construct and the PCR product is purified as described above. The HAT-hVPAC₂ fusion gene PCR product is then utilized for cloning into the pBacPAK8 vector using the BacPAK Baculovirus Expression System from Clontech (Clontech Inc., Palo Alto, CA, USA). The ligation of the HAT-hVPAC₂ fusion gene into the pBacPAK8 vector is essentially as described above. The hVPAC₂/HAT-pBacPAK8 construct is

25 then transfected into TOP10F' competent E. coli cells, ampicillin resistant cells are selected and plasmid DNA is isolated and checked for construct integrity as described above. This construct is then cotransfected with linearized BacPAK6 DNA into Sf21 insect host cells utilizing the CalPhos™ Mammalian Transfection Kit (Clontech Inc., Palo Alto, CA, USA). The insect cells are then incubated for 2-3 days followed by harvest of virus from individual clear plaques. The

30 virus is then amplified in Sf21 cells, the harvested virus titered, and the titered virus used for large scale infection of Sf21 cells utilizing BacPAK Insect Cell Media - all according to the manufacturers recommendations (Clontech Inc., Palo Alto, CA, USA). Recombinant HAT-VPAC₂ fusion protein is then purified using the TALON® CellThru Purification Kit from

Clonotech (Clonotech Inc., Palo Alto, CA, USA) using conditions recommended by the manufacturer. Briefly, infected Sf21 cells are harvested 48 hours after infection and sonicated in extraction/loading buffer. The cell lysate is then put through a TALON® CellThru column. The column is washed twice with extraction/loading buffer and the bound HAT-hVPAC₂ protein is
5 eluted with elution buffer. The eluted protein is analyzed by SDS-PAGE for integrity and protein concentration is quantitated using the Bio-Rad SDS-PAGE system and protein quantitation systems according to the manufacturer's recommendations (Bio-Rad Laboratories, Hercules, Ca). Purified HAT-hVPAC₂ fusion protein is then used for immunizing XenoMouse animals (Abgenix Inc., Fremont, CA) for human monoclonal antibody production as follows. 10 µg of purified
10 recombinant HAT-hVPAC₂ fusion protein in combination with 25 µg of adjuvant monophosphoryl lipid A (Sigma, St. Louis, MO) is used to vaccinate 10 XenoMouse animals multiple times over an eight week period. Serum is obtained from vaccinated animals and utilized in an antigen capture ELISA utilizing purified HAT-hVPAC₂ fusion protein to detect antibodies to the HAT-hVPAC₂ protein by coating polystyrene ELISA plates (Corning Glass Works, Corning, NY) with HAT-
15 hVPAC₂ fusion protein, blocked with PBS-1% BSA, washed and incubated at 37°C for 1 hour with a 1:50 dilution of the serum samples. After washing 5 times with PBS, the plates are incubated at 37°C for 1 hour with alkaline phosphatase-conjugated goat antibodies to human immunoglobulin G. The plates are then washed 5X with PBS and antibodies detected with p-nitrophenyl phosphate substrate (Sigma, St. Louis, MO) in buffer. Optical densities at 405 nm
20 were measured using a plate reader and signal quantitated. Mice with demonstrated high antibody production are used for hybridoma formation. Hybridomas are generated by fusion of splenic cells from the XenoMouse animals with nonsecreting myeloma cell line NSA-bcl 2 using a 4:1 ratio of spleen cells to NSA-bcl2 cells in the presence of 30% polyethylene glycol PEG1450. Fused cells are individually cloned by limiting dilution into 96 well plates and cultured in RPMI-1640 medium
25 containing 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, L-glutamine, 100 u/ml penicillin-streptomycin and hypoxanthine-aminopterin-thymidine (all from Life Technologies, Rockville, MD). Supernatants from the hypoxanthine-aminopterin-thymidine selected hybridomas were screened for human antibody production by ELISA as described previously. Hybridomas which produce human antibodies to the HAT-hVPAC₂ fusion protein are selected for large scale
30 antibody production. Monoclonal antibodies are purified by Protein G-Sepharose chromatography. Briefly, the supernatant from cultured hybridoma clones is loaded onto a Protein G-Sepharose column (SIGMA, St. Louis, MO) in loading buffer, washed 3 times and the IgG is eluted with elution buffer. These antibodies are then used for screening to evaluate hVPAC₂ activation

(agonism) potential. This is accomplished using the methodology as outlined in Example 3. Those human monoclonal antibodies which demonstrate agonist activity toward the hVPAC₂ receptor are designated candidate compounds.

- 5 Example 8. Therapeutic treatment of skeletal muscle atrophy using a human antibody that is an agonist of the VPAC₂ receptor.

A human male subject weighing 50 kg and having significant muscular atrophy of the arms and legs due to prolonged bed rest, is treated to reverse the skeletal muscle atrophy. Once each week for a period of 3 months, 15 mls of an aqueous solution of pH 6.0 comprising the anti-VPAC₂ receptor is administered to the subject via intravenous injection. The solution comprises the following:

	<u>Component</u>	<u>Concentration (mg/ml)</u>
	VPAC ₂ receptor agonist antibody	20
15	L-histidine HCl	0.47
	L-histidine	0.3
	α , α -trehalose dihydrate	20
	Polysorbate 20	0.1
	Bacteriostatic Sterile water	qs to 1 mL

20

At the end of the treatment period, the subject exhibits measurable increases of muscle mass, strength and mobility of the arms and legs.

- 25 Example 9. Prophylactic treatment of skeletal muscle atrophy using a human antibody that is an agonist of the VPAC₂ receptor.

A human female subject weighing 55 kg is scheduled for hip joint replacement surgery in one month. The subject is treated to enhance skeletal muscle mass prior to and following surgery to ultimately reduce the level of skeletal muscle atrophy due to muscle disuse during post-surgery recovery. Specifically, once each week for a period of 1 month prior to surgery and for 2 months post-surgery, 18 ml of an aqueous solution of pH 6.0 comprising the anti-VPAC₂ receptor is administered to the subject via intravenous injection. The solution comprises the following:

30

	<u>Component</u>	<u>Concentration (mg/ml)</u>
	VPAC activating antibody	20
	L-histidine HCl	0.47
	L-histidine	0.3
5	α , α -trehalose dihydrate	20
	Polysorbate 20	0.1
	Bacteriostatic Sterile water	qs to 1 mL

At the end of the treatment period, the subject exhibits measurable preservation of muscle mass, strength and mobility of the arms and legs as compared to the subject's expected status without antibody therapy.

Example 10. Prophylactic treatment of skeletal muscle atrophy using a human antibody that is an agonist of the VPAC₁ receptor.

15 A human female subject weighing 45 kg undergoes a casting procedure to treat a simple fracture of the humerus after a fall. The subject is treated to prevent atrophy of the skeletal muscle of the affected arm and shoulder due to disuse and limited use during fracture healing. Specifically, once each week starting on the day of casting, 13 ml of pH 6.0 comprising the anti-VPAC₁ receptor is administered to the subject via intravenous injection. The solution comprises the following:

	<u>Component</u>	<u>Concentration (mg/ml)</u>
	VPAC ₁ activating antibody	20
	L-histidine HCl	0.47
	L-histidine	0.3
	α , α -trehalose dihydrate	20
20	Polysorbate 20	0.1
	Bacteriostatic Sterile water	qs to 1 mL

At the end of the treatment period, the subject exhibits measurable preservation of muscle mass, strength and mobility of the affected arm and shoulder and a reduced course of physical therapy as compared to the subject's expected status and follow-up treatment without antibody therapy.

Example 11. Prophylactic treatment of skeletal muscle atrophy using an agonist of the VPAC₂ receptor, Ro 25-1553.

A human female subject weighing 50 kg is admitted to the hospital in a comatose state. The subject is treated to prevent atrophy of the skeletal muscle of the entire body due to disuse while in the comatose state. Specifically, once each month while in the coma, the subject is administered, via intramuscular injection, 3 ml of an aqueous solution of pH 6.0 comprising the following:

	<u>Component</u>	<u>Concentration (mg/ml)</u>
10	Ro 25-1553 (VPAC agonist)	500
	D,L lactic and glycolic acid copolymer	5

As a result of treatment, the subject exhibits measurable preservation of skeletal muscle mass and reduced physical therapy needs during the coma and after regaining consciousness, as compared to the subject's expected status without drug therapy.

The present invention is not to be limited in scope by the specific embodiments described which are intended solely as illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. These include, but are not limited to, species of test animal, nature and type of VPAC agonists, sex of the animal, model of atrophy, method of activating VPAC including genetic methodologies, etc. Various modifications of the invention, in addition to those shown and described herein will be apparent to those skilled in the art upon reading foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

All references herein are incorporated herein by reference.

What is claimed is:

1. A method for identifying candidate compounds for regulating skeletal muscle mass or function, comprising:

- (a) contacting a test compound with a VPAC receptor; and
- (b) determining whether the test compound binds to the VPAC receptor, wherein test compounds that bind to the VPAC receptor are identified as candidate compounds for regulating skeletal muscle mass or function.

2. A method for identifying candidate therapeutic compounds for regulating skeletal muscle mass or function, comprising:

- (a) contacting a test compound with a VPAC receptor;
- (b) determining whether the test compound binds to the VPAC receptor; and
- (c) administering a test compound determined, in step (b), to bind to the VPAC receptor, to a non-human animal, and determining whether the test compound regulates skeletal muscle mass or function in the animal, wherein test compounds that regulate skeletal muscle mass or function in the animal are identified as candidate therapeutic compounds for regulating skeletal muscle mass or function *in vivo*.

3. A method for identifying candidate compounds for regulating skeletal muscle mass or function, comprising:

- (a) contacting a test compound with a cell expressing a VPAC receptor, and
- (b) determining whether the test compound activates the VPAC receptor, wherein test compounds that activate the VPAC receptor are identified as candidate compounds for regulating skeletal muscle mass or function.

4. The method for identifying candidate compounds according to Claim 3, wherein the VPAC receptor is expressed on a eukaryotic cell and is a functional VPAC₁ receptor.

5. The method for identifying candidate compounds according to Claim 3, in which the VPAC receptor is expressed on a eukaryotic cell and is a functional VPAC₂ receptor.

6. The method for identifying candidate compounds according to Claim 3, the cell having a cellular cAMP level, in which determining whether the test compound activates the VPAC receptor involves measuring the cellular cAMP level.

7. The method for identifying candidate compounds according to Claim 6, in which the cell further comprises a reporter gene operatively associated with a cAMP responsive element and measuring the cellular cAMP level involves measuring expression of the reporter gene.

8. The method for identifying candidate compounds according to Claim 7, in which the reporter gene is alkaline phosphatase, chloramphenicol acetyltransferase, luciferase, glucuronide synthetase, growth hormone, placental alkaline phosphatase or Green Fluorescent Protein.

9. A method for identifying candidate therapeutic compounds for regulating skeletal muscle mass or function, comprising:

- (a) contacting a test compound with a cell which expresses a functional VPAC receptor;
- (b) determining whether the test compound activates the VPAC receptor; and
- (c) administering a test compound determined, in step (b), to activate the VPAC receptor,

to a non-human animal, and determining whether the test compound regulates skeletal muscle mass or function in the animal, wherein test compounds that regulate skeletal muscle mass or function in the animal are identified as candidate therapeutic compounds for regulating skeletal muscle mass or function *in vivo*.

10. A method for identifying candidate therapeutic compounds from a group of one or more candidate compounds which have been previously determined to activate the VPAC receptor comprising:

administering a candidate compound to a non-human animal, and determining whether the candidate compound regulates skeletal muscle mass or function in the treated animal, wherein candidate compounds that regulate skeletal muscle mass or function in the treated animal are identified as candidate therapeutic compounds for regulating skeletal muscle mass or function *in vivo*.

11. A method for identifying candidate compounds that prolong or augment the activation of a VPAC receptor or of a VPAC receptor signal transduction pathway, comprising:

- (a) contacting a test compound with a cell which expresses a functional VPAC receptor;

(b) treating the cell with an agonist for a sufficient time and at a sufficient concentration to cause desensitization of the VPAC receptors in control cells; and

(c) determining the level of activation of the VPAC receptor, wherein test compounds that prolong or augment the activation of a VPAC receptor or of a VPAC receptor signal transduction pathway are identified as candidate compounds for regulating skeletal muscle mass or function.

12. The method for identifying candidate compounds that prolong or augment the activation of VPAC receptors according to Claim 11 wherein step (b) is preformed before or concurrently with step (a).

13. A method for identifying candidate compounds for increasing VPAC receptor expression, comprising:

(a) contacting a test compound with a cell or cell lysate containing a reporter gene operatively associated with a VPAC receptor regulatory element; and

(b) detecting expression of the reporter gene wherein test compounds that increase expression of the reporter gene are identified as candidate compounds for regulating skeletal muscle mass or function.

14. A method for identifying candidate compounds for increasing the expression of VIP or a VIP analog, comprising:

(a) contacting a test compound with a cell or cell lysate containing a reporter gene operatively associated with a VIP analog regulatory element; and

(b) detecting expression of the reporter gene wherein the test compounds that increase expression of the reporter gene are identified as compounds for regulating skeletal muscle mass or function.

15. A pharmaceutical composition, comprising:

(a) a safe and effective amount of a VPAC receptor agonist; and

(b) a pharmaceutically-acceptable carrier.

16. A method for increasing skeletal muscle mass or function in a subject in which such an increase is desirable, comprising:

(a) identifying a subject in which an increase in muscle mass or function is desirable; and

- (b) administering to the subject a safe and effective amount of compound selected from the group consisting of a VPAC receptor agonist, a compound that prolongs or augments the activation of VPAC receptors or the activation of a VPAC receptor signal transduction pathway, an expression vector encoding a functional VPAC receptor, an expression vector encoding a constitutively active VPAC receptor, a compound that increases expression of VPAC receptors, a compound that increases expression of VIP and a compound that increases expression of a VIP analog.

17. The method for increasing skeletal muscle mass or function according to Claim 16 wherein the compound is a VPAC receptor agonist and the VPAC receptor agonist is VIP, PACAP-27, PACAP-38, helodermin, peptide histidine isoleucine amide, peptide histidine methionine amide, peptide histidine valine amide, growth hormone releasing hormone, secretin, glucagon, (Arg15, Arg21) VIP, [Arg15,20,21Leu17]-VIP-Gly-Lys-Arg-NH₂, [K¹⁵, R¹⁶, L²⁷,VIP(1-7), GRF(8-27)-NH₂], multimeric VIP fusion protein, Ro 25-1553, Ro 25-1392 or PACAP(6-38).

18. A method for treating skeletal muscle atrophy in a subject in need of such treatment, comprising:

- (a) identifying a subject in need of treatment for skeletal muscle atrophy; and
- (b) administering to the subject a safe and effective amount of compound selected from the group consisting of a VPAC receptor agonist, a compound that prolongs or augments the activation of VPAC receptors or the activation of a VPAC receptor signal transduction pathway, an expression vector encoding a functional VPAC receptor, an expression vector encoding a constitutively active VPAC receptor, a compound that increases expression of VPAC receptors, a compound that increases expression of VIP and a compound that increases expression of a VIP analog.

19. A method for treating skeletal muscle atrophy according to Claim 18, wherein the compound is a VPAC receptor agonist.

20. The method for treating skeletal muscle atrophy according to Claim 19, wherein the VPAC receptor agonist is identified by a method for identifying candidate compounds for regulating skeletal muscle mass or function, comprising:

- (a) contacting a test compound with a VPAC receptor, and

(b) determining whether the test compound activates the VPAC receptor, wherein test compounds that activate the VPAC receptor are identified as candidate compounds for regulating skeletal muscle mass or function.

21. The method for treating skeletal muscle atrophy according to Claim 19, wherein the VPAC receptor agonist is a chimeric or human antibody.

22. The method for treating skeletal muscle atrophy according to Claim 19, further comprising: administering to the subject a safe and effective amount of a compound that prolongs or augments the activation of VPAC receptors or the activation of a VPAC receptor signal transduction pathway.

23. The method for treating skeletal muscle atrophy according to Claim 19, wherein the VPAC receptor agonist is VIP, PACAP-27, PACAP-38, helodermin, peptide histidine isoleucine amide, peptide histidine methionine amide, peptide histidine valine amide, growth hormone releasing hormone, secretin, glucagon, (Arg15, Arg21) VIP, [Arg15,20,21Leu17]-VIP-Gly-Lys-Arg-NH₂, [K¹⁵, R¹⁶, L²⁷,VIP(1-7), GRF(8-27)-NH₂], multimeric VIP fusion protein, Ro 25-1553, Ro 25-1392 or PACAP(6-38)..

24. A purified antibody specific for a VPAC receptor, wherein the antibody is a chimeric or human antibody.

25. The antibody of Claim 24, wherein the antibody is an agonist of a VPAC receptor

26. The antibody of Claim 24, wherein the antibody is a human antibody.

[illegible][illegible]

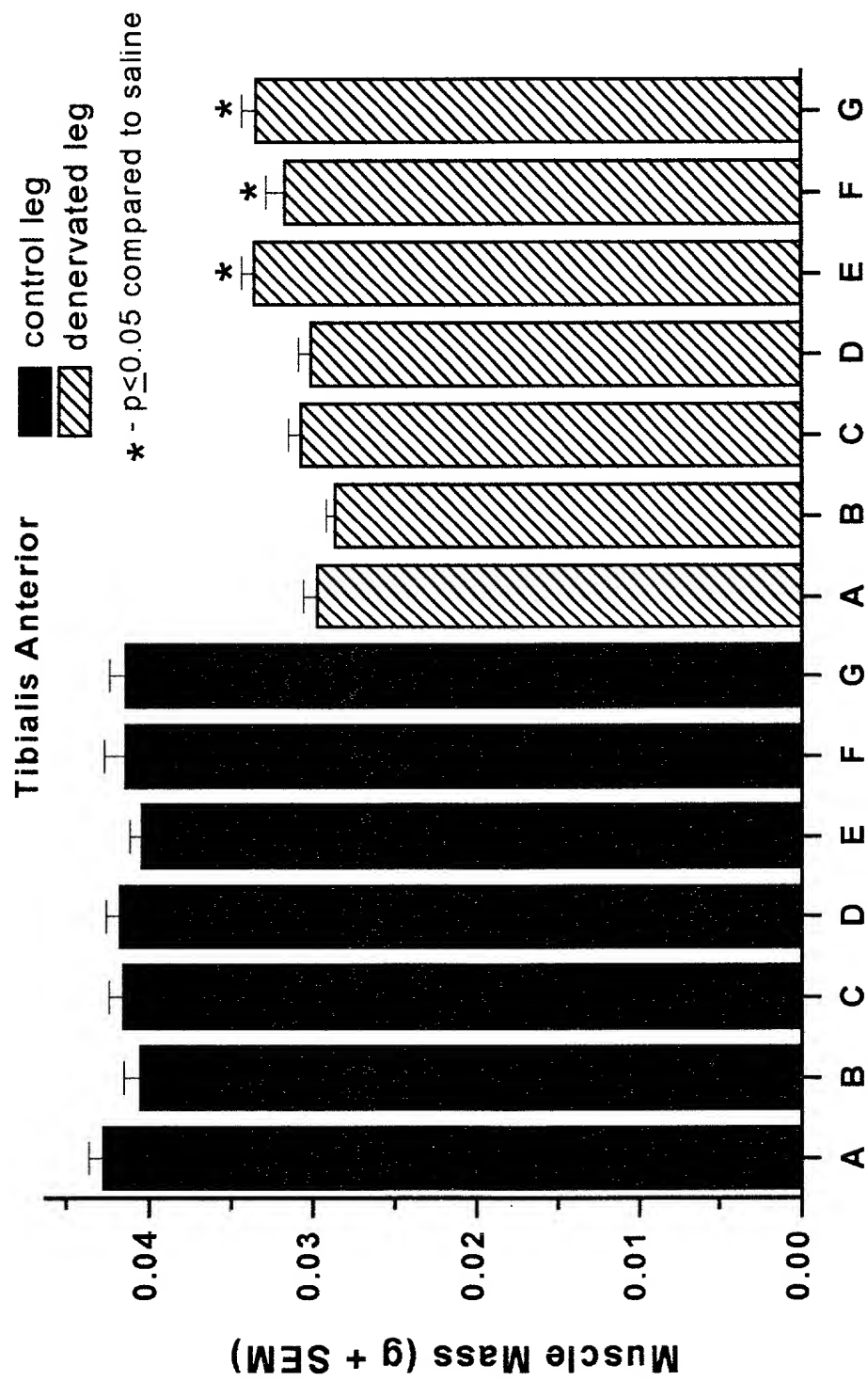


Fig. 1A

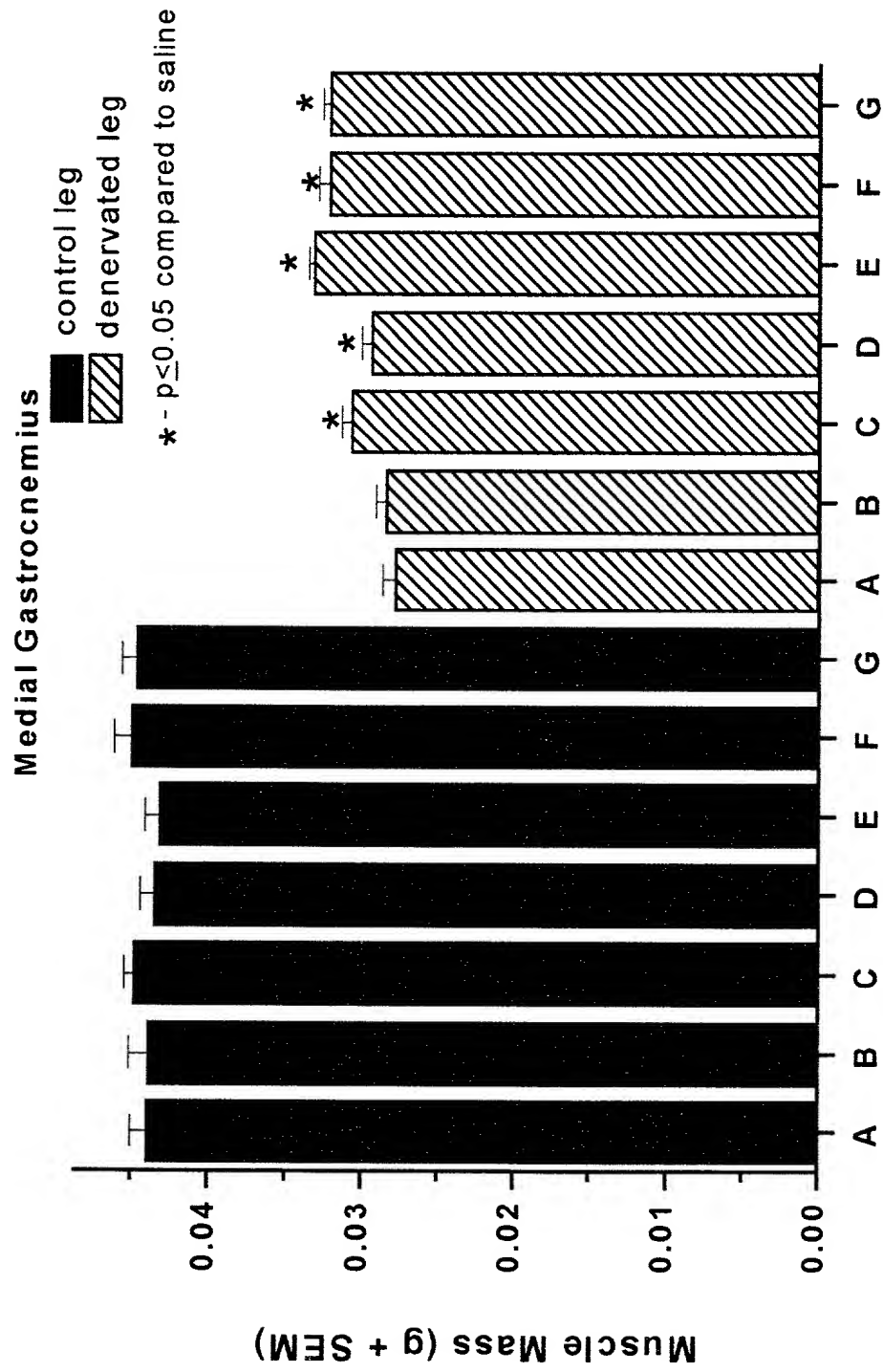


Fig. 1B

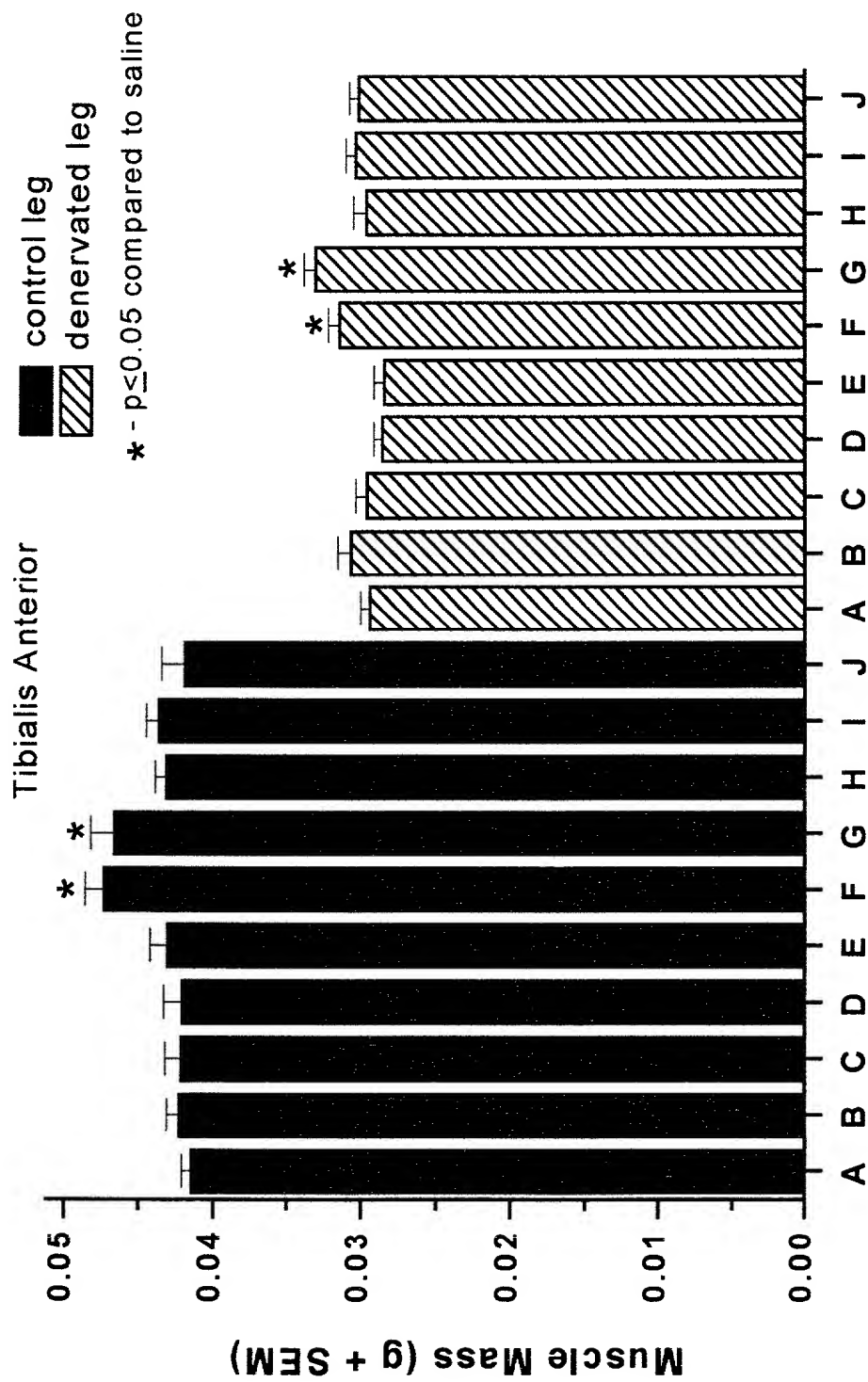


Fig. 2A

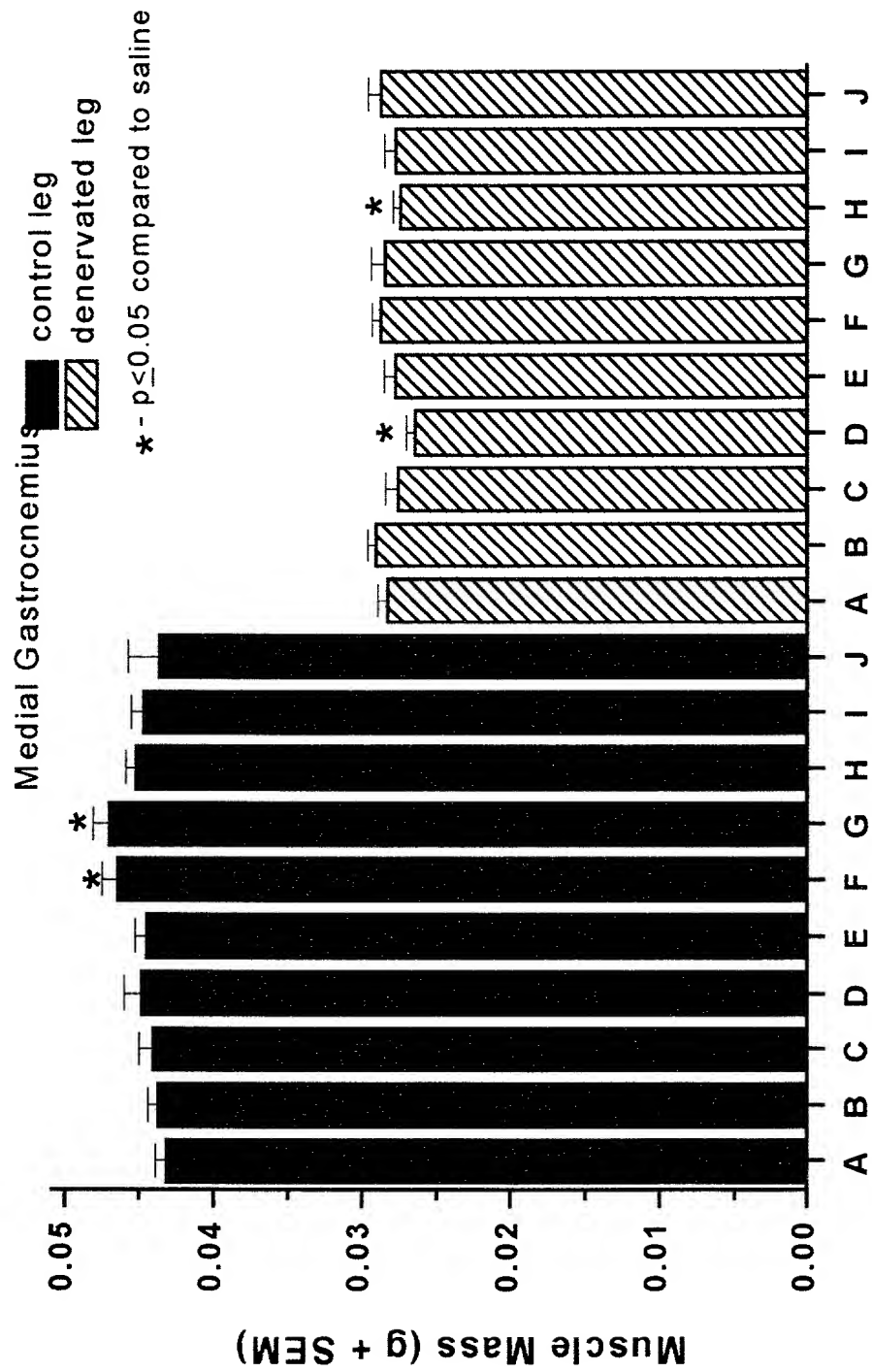


Fig. 2B

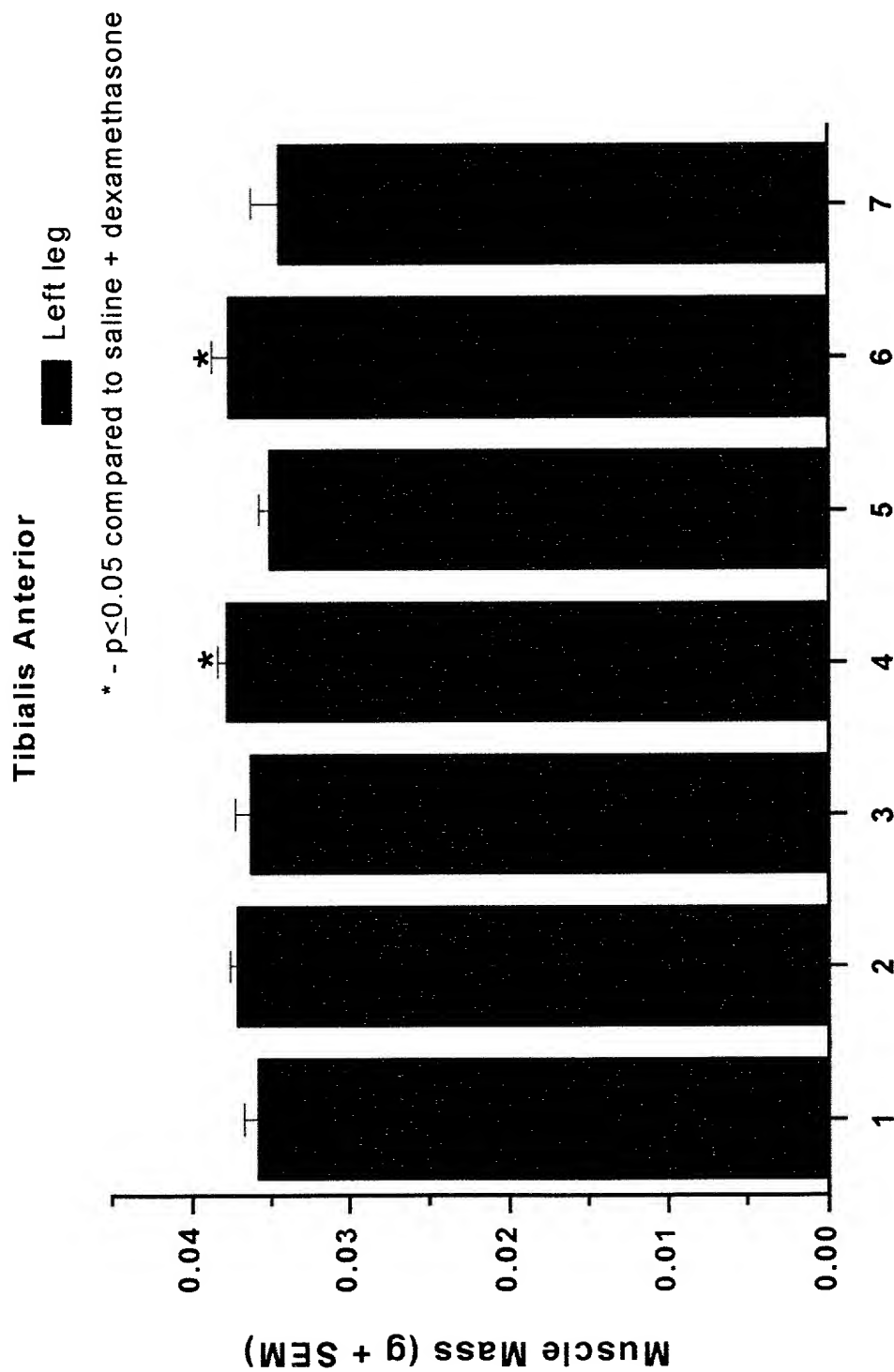


Fig. 3A

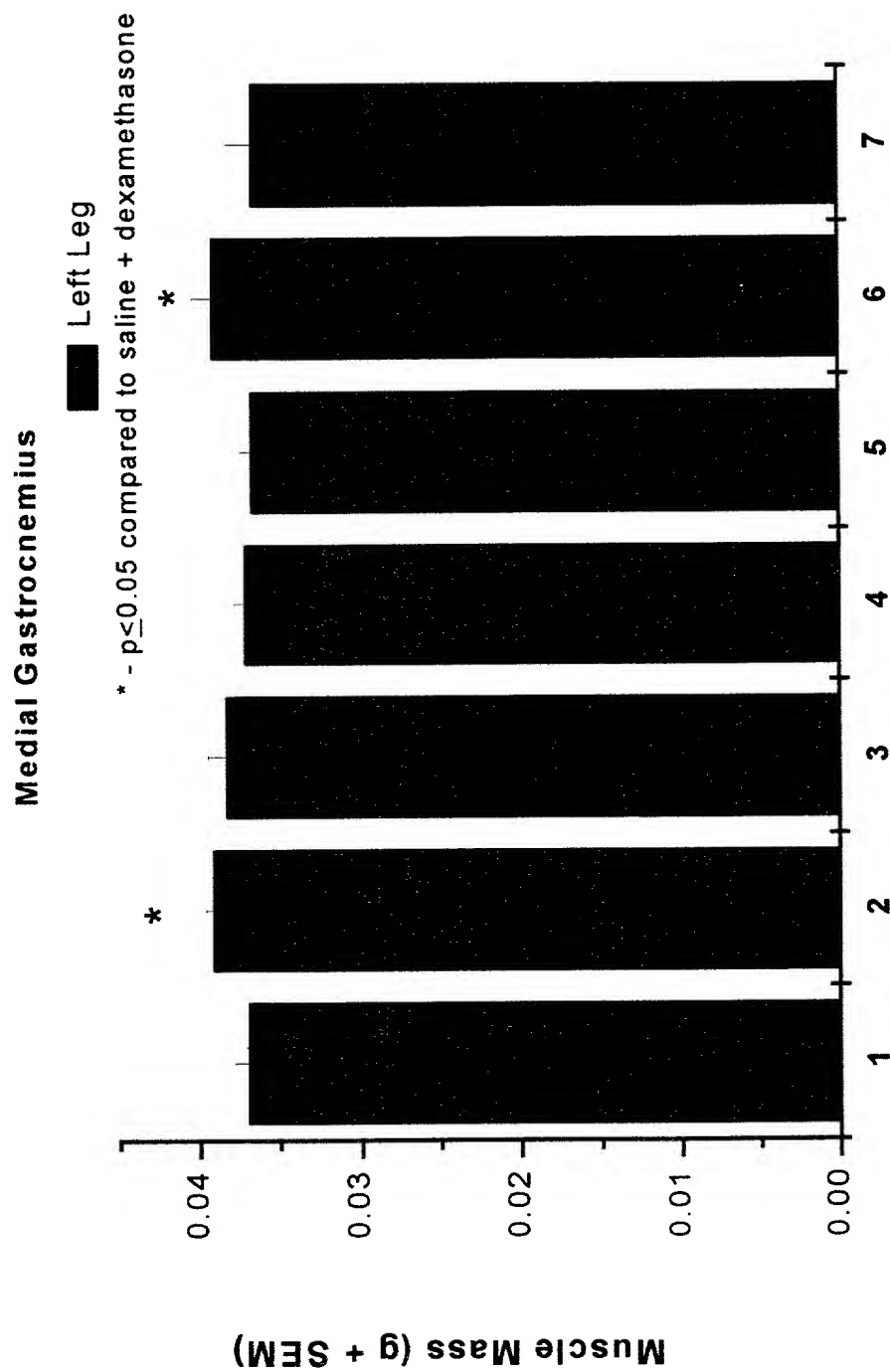
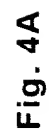


Fig. 3B

Muscle Mass (g \pm SEM)



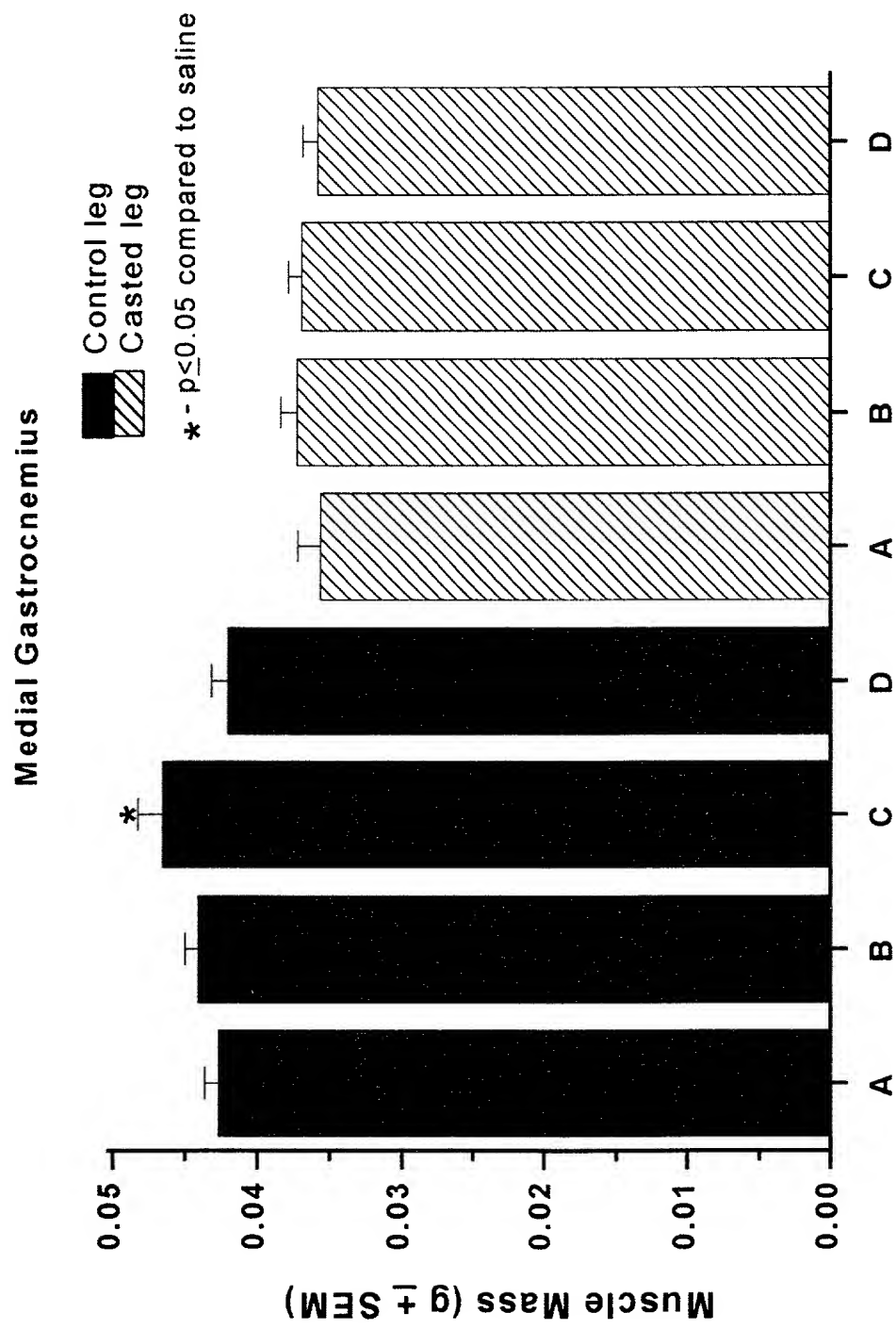


Fig. 4B

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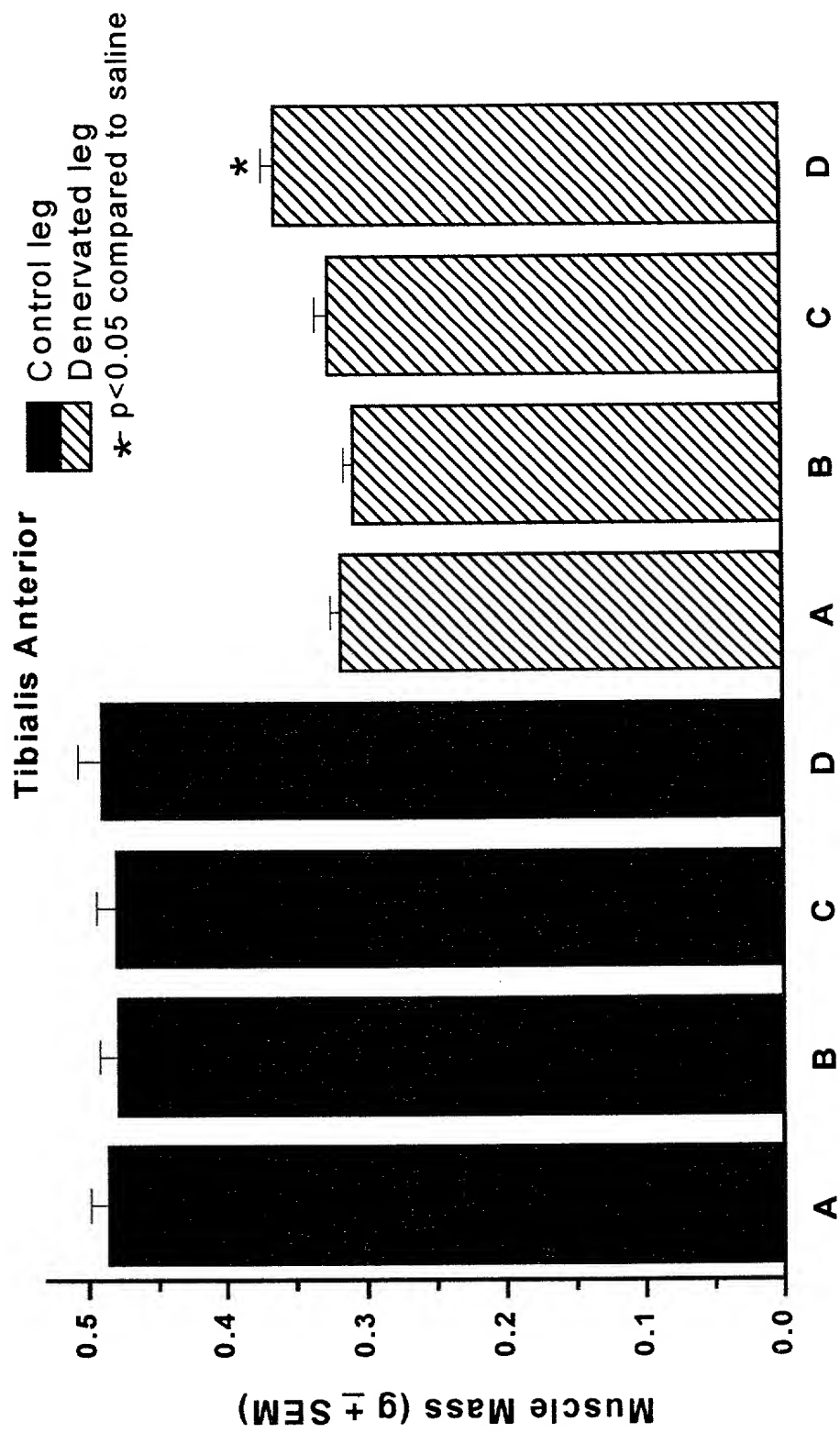


Fig. 5A

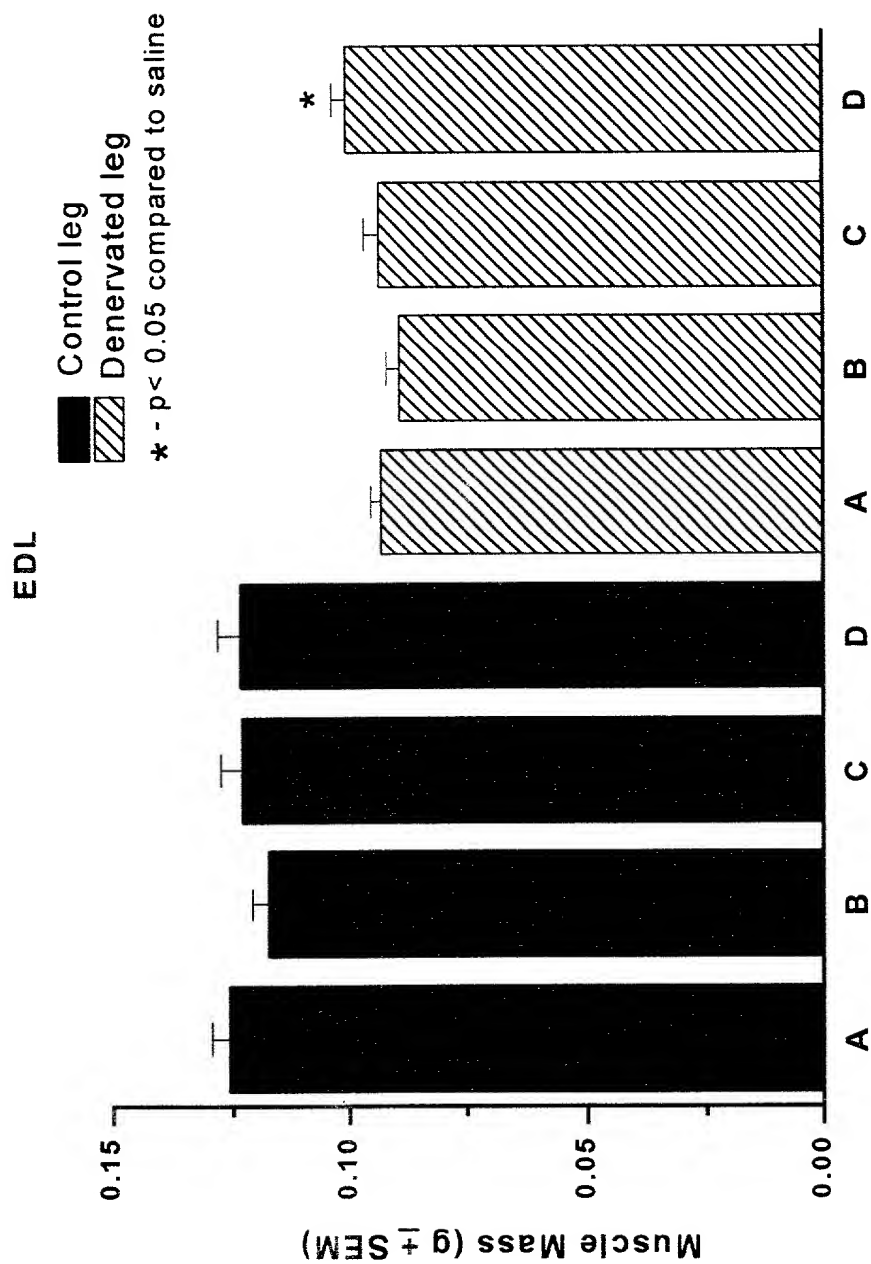


Fig. 5B

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled Methods For Identifying Compounds for Regulating Muscle Mass or Function Using Vasoactive Intestinal Peptide Receptors the specification of which

(check one) ☒ is attached hereto.
☐ was filed on _____ as United States Application No. or
PCT International Application Serial No. _____
and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations §1.56.

I hereby claim foreign priority benefits under Title 35 United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
(Number)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/> Yes	<input type="checkbox"/> No

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Serial No.	Filing Date	Application Serial No.	Filing Date
------------------------	-------------	------------------------	-------------

I hereby claim the benefit under Title 35 United States Code §120 of any United States application(s), or §365(c) of any PCT International application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35 United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (If applicable)

As named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Atty Name	Atty Reg Number.	Associate Power of Attorney Attached	
Cynthia M. Bott	46,568	<input type="checkbox"/> Yes	<input checked="" type="checkbox"/> No
James C. Kellerman	43,708	<input type="checkbox"/> Yes	<input checked="" type="checkbox"/> No
Carl J. Roof	37,708	<input type="checkbox"/> Yes	<input checked="" type="checkbox"/> No
Steven W. Miller	31,984	<input type="checkbox"/> Yes	<input checked="" type="checkbox"/> No
Karen F. Clark	32,974	<input type="checkbox"/> Yes	<input checked="" type="checkbox"/> No
T. David Reed	32,931	<input type="checkbox"/> Yes	<input checked="" type="checkbox"/> No
Timothy B. Guffey	41,048	<input type="checkbox"/> Yes	<input checked="" type="checkbox"/> No

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature _____

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